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Synthesis of capillaries with inorganic salts for OTCEC separation

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SYNTHESIS OF CAPILLARIES WITH INORGANIC SALTS FOR OTCEC SEPARATION

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ABSTRACT

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By

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Open-tubular electrochromatography (OTCEC) involves the modification of the inner-wall surface of the capillary, as a means of improving sample separation. The inner wall of the capillary is etched before bonding with a C-18 group. The etching process is carried out in the presence of inorganic salts. Later, the capillaries are modified by bonding an octadecyl (C-18) group. The capillaries are evaluated using the separation of enkephalins and heterocyclic aromatic amines. Three evaluations are made: 1) the effect of the presence of the inorganic salts; 2) the effect of pH on the electroosmotic flow (EOF); 3) and the effect of an organic moiety on the surface. The best separations are observed with the capillary etched in the absence of salts and then C-18 modified, and with the capillaries etched in the presence of chromium chloride ($CrCl_3$), ammonium carbonate ($(NH_4)_2CO_3$) and cupric chloride ($CuCl_2$) salts and subsequently chemically modified.

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1. INTRODUCTION

1.1 Chromatography

Chromatography is a separation technique that finds applications in all branches of science. It involves the separation of analytes of interest from a broad range of chemicals dissolved together in the same solution. Tswett first introduced chromatography where the separation process took place in glass tubes (1). The continuous development in the technique has modified it from a slab model to column and to chip technologies. The chromatographic separation is carried out by dissolving the sample in the mobile phase which is usually a gas, liquid or supercritical fluid. This mobile phase is forced through an immiscible stationary phase placed in a column or coated on a solid surface. The constituents of the sample distribute themselves between the mobile and stationary phases to varying degrees and are thus separated. Basically, the separation of the analytes in a sample mixture is based on the differences in distribution ratios between the two phases. Of all the various kinds of chromatographic techniques liquid chromatography has become the most important because of the numerous analytes, mobile and stationary phases that can be used.

1.2 Capillary Electrophoresis

Capillary electrophoresis (CE) is an emerging separation technique similar to chromatography. Since its introduction in the late 1960's (2), CE has developed into a powerful separation method today. Its minimal sample

and solvent requirement has allowed CE to gain popularity as an important separation technique (3). In its simplest form, it is a version of the original Tisileus method where the migration of analytes takes place within an electrolyte solution under the influence of an electric field. Its popularity is based not only on its simplicity but also on the additional advantages of speed, versatility and low running costs. With the recent interest in the genome project, CE is also considered as a promising technique for protein separation due to its high resolution, sensitivity and above all its small-scale sample requirement. In CE samples are separated in narrow-bore capillaries (20 to 100 μm i.d.) in the presence of an electric field. Unlike HPLC the analytes in CE are driven by electrophoresis that is developed when an electric field is applied.

CE itself is a family of different separation methods including capillary zone electrophoresis (CZE), capillary electrochromatography (CEC), micellar electrokinetic capillary chromatography (MEKC), capillary isoelectric focusing (CIEF), and isotachophoresis. Among these techniques CEC will be emphasized since it is the method used in this study. However, it is important to discuss the theory of CE as it aids in understanding the fundamental principles of CEC.

The theory that governs electrophoresis is directly applicable to CE and is dealt with briefly here. Electrophoresis is the migration of electrically charged particles under the influence of an electric field. The first successful

electrophoretic analyses in free solution were performed by Hjerten in 1958 (4). The basic concept of CE is based on sample separation by differential migration of charged species under the influence of an applied voltage. The migration of sample ions under the influence of an applied electric field is called electrophoretic mobility. The difference in the migration times is based on the amount of charge the ion carries and its hydrodynamic mass. Thus the velocity of the ion is proportional to the strength of the electric field applied and the electrophoretic mobility.

$$v = \mu_e E \qquad \text{Eq (1)}$$

Where v = velocity of the ion

μ_e = electrophoretic mobility

E = applied electric field

As the ions have to move in an aqueous solution the viscosity of the medium also plays an important role. The velocity of the ion is inversely proportional to the viscosity. The equation governing the speed of the ion mobility is given as

$$\mu_e = q / 6\pi\eta r \qquad \text{Eq (2)}$$

Where q = ion charge

η = solution viscosity

r = ion radius

The main components of a CE system include a capillary, buffer reservoirs, a detector, and a power supply (Figure 1). Both ends of the

capillary are immersed in two identical buffer reservoirs one of which has a cathode and the other an anode. A detector is placed at some point between the two reservoirs. Solvent will move from the anode side (inlet reservoir) to the cathode side (outlet reservoir) through the capillary by electroosmosis.

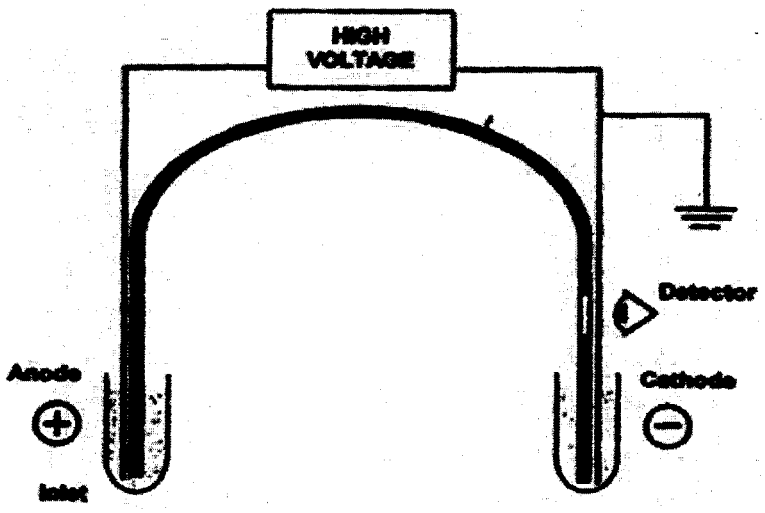


Figure 1 – Capillary Electrophoresis System

The length of the capillary from the anode end to the detector is critical as this is the distance where the sample ions migrate and separate. A capillary that is too short doesn't give good resolution of the peaks and a capillary that is too long results in diffusion and long migration times. The polyimide coating is taken off a few centimeters from the outlet end exposing the glass wall. This area acts as a flow cell through which the sample is detected. When voltage is applied the positively charged species in the

sample will move towards the cathode, which are detected in the order of their migration. The migration time of the species depends on the charge they possess and their mass. During their course of movement the species also interact with the silica surface of the capillary. The silanol groups of the silica surface that are not hydrogen bonded to neighboring -OH groups are negatively charged. These groups ionize in the aqueous buffer when the pH reaches 3 or above. Positively charged species are attracted to this negatively charged surface (Figure 2). An excess of positive ions accumulate along the

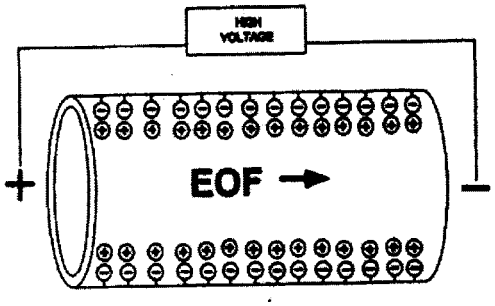


Figure 2 – Electroosmotic Flow Direction of the Capillary Electrophoresis System

surface forming a double layer. The fixed inner layer is called the Stern or Helmoltz layer (Figure 3). The outer mobile layer called the Guoy-Chapman layer, which tends to move towards the cathode causes solvent flow. This bulk movement of the solvent along with the solute species under the influence of an electric field is referred to as electroosmotic flow (EOF).

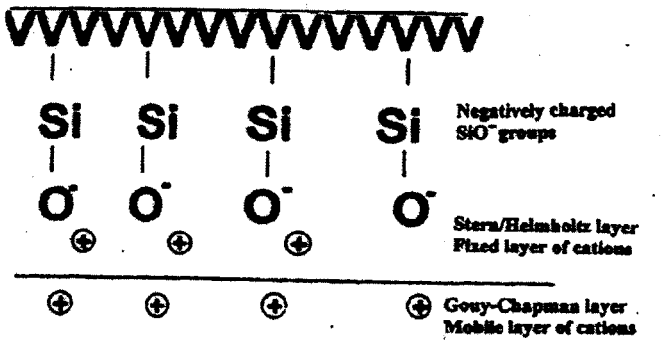


Figure 3 – Double Layer on the Inner Surface of Capillary

The silanoate groups on the inner wall of the capillary attract the cations forming a fixed layer, while the mobile layer of cations is pulled towards the cathode resulting in electroosmotic flow.

One of the advantages of EOF and electrophoretic migration is "plug like flow" in CE contrary to HPLC where a "laminar flow" is seen (Figure 4). In HPLC the solvent is driven by pressure which pushes the center of the mobile phase faster than the sides; the solvent-wall interactions slow down

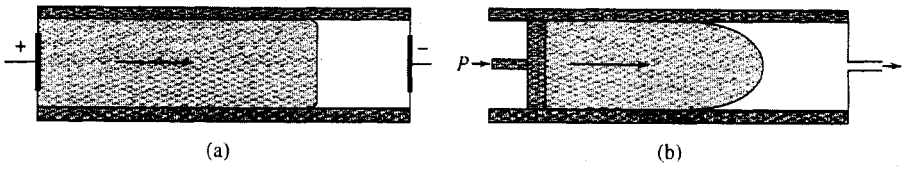


Figure 4 – Flow Profiles for Liquids Under (A) Electroosmotic Pressure and (B) Hydrodynamic Pressure.

the movement on the sides creating a bow like or a parabolic shape. Whereas the driving force of the flow is uniformly distributed in CE without any

pressure drop. Because of the EOF a flat front is created. The flat front results in less band diffusion.

Another benefit of EOF is that its magnitude is large enough to cause the movement of all solutes towards the cathode regardless of their charge. This migration of all cations and anions is called electrophoretic mobility. The electrophoretic mobility of each species differs. Cations tend to move faster along with EOF towards the cathode. Anions migrate a little later as they tend to move towards the anode and neutral species move at the pace of the EOF. The presence of the sample ions can be detected by UV absorbance in the order the analyte ions pass the window. The detector sends a signal for further data analysis converting electronic signals to sample peaks. The resulting output of the sample peaks is called an electropherogram.

While EOF is usually beneficial, it sometimes needs to be controlled. At high pH, the EOF can be too fast which results in elution of solutes before a separation has occurred. Conversely, at low or moderate pH, the EOF will be low resulting in much longer times for the separation process to be carried out. Basically, EOF can be controlled by changing the buffer viscosity, lowering the electric field or changing the capillary surface (5). The first two options have their own disadvantages with regard to ionization of the solute, efficiency and resolution. The most effective way seems to be changing the capillary surface.

1.3 Capillary Electrochromatography

CEC, first introduced by Pretorius et al (6), is a hybrid technique of HPLC and CE. As in HPLC, CEC has a stationary phase in the capillary and as in CE it uses EOF to drive the solvent. The stationary phase can be changed to improve the separation of various sample species. The EOF gives a flat profile to the solvent flow resulting in high efficiency and less band diffusion. The separation takes place both by electrophoretic mobility and solute interaction with the stationary phase. CEC uses a CE system but with a modified capillary. CEC can utilize either packed or open tubular capillaries. Depending on the mode of the stationary phase used CEC is divided into two main types: packed-column CEC (P-CEC) (7) and open-tubular CEC (OTCEC) (8).

1.3.1 Packed Capillary Electrochromatography

In P-CEC the capillary is packed with the solid material confined between two frits. The capillary is packed with porous silica beads with attached organic groups (e.g. C8, C18). The separation mechanism is based on the interaction between the solute and the bonded stationary phase. The solvent is driven by EOF that develops under the influence of the applied electric field. The solute species are also driven by EOF but at the same time the electrophoretic mobility of the individual species influences their migration time. The advantage of P-CEC is that there is no pressure drop which aids in using small size particles to decrease the eddy diffusion factor.

There are at least three known packing procedures. One way is similar to the method used for packing ordinary HPLC columns by applying pressure through a pneumatic pump (9). The second method is making a drawn packed capillary. Dried silica is placed in a narrow glass tube of 1 or 2 mm that is closed at one end. This glass tube is drawn to the desired diameter. But the drawback with this method is that only bare silica can be packed with this procedure (10). The third method is electrokinetic packing. The packing material is suspended in an appropriate solvent that is well sonicated. The slurry of the packing material is electrokinetically moved by subjecting it to an appropriate voltage for about 1hour (11). Then the sintering process is used to fabricate the frits on the column.

There are several examples of reported separations like hydrocarbons, large PAHs, fullerenes and other solutes that suggest that CEC is a versatile technique with analytical features unique from HPLC and CE (12). In this technique it is possible to apply both a pressure gradient and an electric field across a packed capillary. This could be applied to separate both ionic and nonionic compounds (13). The concept of solvent gradient that is usually employed in HPLC is also applicable to CEC that is achieved by mixing the flow from two different solvent reservoirs under the influence of high voltages (14). But there are some fundamental problems with P-CEC. One shortcoming of P-CEC is that packing the capillary is a difficult process due to the small diameter of the inner bore. The other shortcoming of this

method is the making of frits which leads to bubble formation and disturbance of the packed bed resulting in the loss of efficiency. The other thing is that the frits are made *in situ* which leads to non-identical frits for each capillary. These drawbacks are overcome by the OTCEC approach.

1.3.2 Open Tubular Capillary Electrochromatography

The stationary phase in OTCEC involves the modification of the inner surface of the capillary. It is achieved in various ways. It can be a temporary or a permanent modification. Temporary modifications are done by using adsorbents that adhere to the inner surface of the capillary that can be removed afterwards. Similarly, the inside of the capillaries are coated with some surfactants, or polymers that can be either hydrophobic or hydrophilic in order to block access to the silanol groups. Addition of modifiers to the running buffers is referred to as dynamic modification.

The separation of biomolecules like proteins and peptides is not successful with a bare capillary because of solute wall interactions. In order to minimize wall interactions for proteins which are hydrophilic, a hydrophobic coating can be applied and vice versa. Capillaries coated with polyacrylamide have been very successful in achieving good protein separations (15). Various types of polyethers and diol moieties are also effective hydrophilic coatings that have been as successful as polyacrylamide (16). The concern with the wall coatings is that the phases should be stable over many injections in order to have reproducible quantitative analyses.

The other process by which the problems of a bare capillary and the instability of a bonded coating can be addressed involves the attachment of an organic moiety in contact with a surfactant (17). In this method an organic moiety is first attached to a surfactant and another surfactant is physically adsorbed on this hydrophobic surface. The hydrophobic part of the surfactant adheres to the octadecyl moiety and the polar group of the adsorbed molecule gives a hydrophilic surface. But the hydrophilic surface is subject to degradation during the electrophoresis process through desorption.

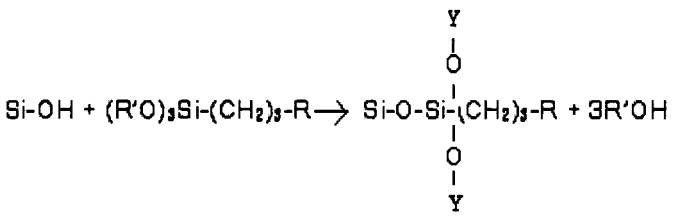
Polymer coated-columns are beneficial in the respect that the surface is generally well-shielded from the solutes during their course of travel through the capillary. But most polymer procedures involve a multi-step process that can be time consuming and experimentally difficult (18).

Permanent modifications involve chemical bonding of an organic moiety of interest to the silanol groups. This avoids the making of frits. But the greatest disadvantage is the small surface area for the solute to interact with the stationary phase. This is overcome by etching the inner surface of the capillary before modification, which increases the surface area (19).

There are several approaches to the modification of the capillary inner surface. Some of them that act as the stationary phase are discussed briefly here. The third way of modification is the fabrication of a monolith *in situ* within the column (20).

1.3.2.1 Methods of Modification:

In the case of permanent modification of a capillary wall with an organic compound, several methods have been employed which are fairly successful in minimizing protein adsorption as well as eliminating EOF. Organosilanization is the most common approach by which a fully hydroxylated silica surface is coupled with an organic group, $(R'O)_3$ Si-$(CH2)_3$ -R, to form a siloxane (Si-O-Si-C) linkage, as follows (21).



(3)

where R' may be an alkyl group, R contains the hydrophilic moiety and Y represents hydrogen or a neighboring silicon group. A major drawback of this approach is the unsatisfactory hydrolytic stability of the siloxane linkage under extreme pH conditions. Furthermore, the coverage of the organic moieties on the silica surface is limited by the steric hinderance of the organic group, which ultimately leads to a higher concentration of unreacted surface silanols. Silicon-carbon (Si-C) linkages have long been considered to be more stable towards hydrolysis than a siloxane (Si-O-Si-C) linkage, especially under severe pH conditions (22, 23). This approach involves the sequential reaction of the silica surface with a chlorinating agent (e.g., $SOCl_2$) followed by an organometallic compound (e.g., Grignard reagent).

$$\equiv Si\text{-}OH + SOCl_2 \rightarrow \equiv SiCl + HCl + SO_2 \quad (4)$$

$$\equiv Si\text{-}Cl + R\text{-}MgBr \rightarrow \equiv Si\text{-}R + MgBrCl \quad (5)$$

In addition to enhanced stability, a denser coverage of organic functionalities is thought to be formed on the silica surface (24, 25). Despite the hydrolytic advantage provided by the direct Si-C linkage, bonded capillaries prepared by this approach have not reached wide spread use as they are more difficult to prepare than the organosilanized counterpart.

An alternative approach to produce Si-C linkages on silica surfaces involves the formation of a hydride bond (Si-H) to which an organic moiety is added (26). This approach is dealt with in detail as this method was used in this investigation. But before these two steps, etching of the capillaries was performed. The process of etching prior to modification creates a new chemical composition in the inner wall of the capillary that affects both EOF as well as the adsorptive properties of the capillary (27). The different methods of modification are shown in Figure 5.

Bonding of the organic moiety to the etched capillaries utilizes the silanization / hydrosilation process. The etched surface of the capillary is first treated with triethoxysilane (TES) to produce a hydride layer. The reaction with TES results in a monolayer deposited on the surface so most of the silanols are hydrides.

$$- Si - OH + (OEt)_3\ Si - H \rightarrow - Si - O - Si - H + 3EtOH \quad (5)$$

REACTION TYPE	REACTION	SURFACE LINKAGES
ESTERIFICATION	$Si - OH + R - OH \rightarrow Si - OR + H_2O$	Si - O - C
ORGANOSILANE	$Si - OH + X - SiR'_2R \rightarrow Si - O - SiR'_2R + HX$	Si - O - Si - C
CHLORINATION FOLLOWED BY REACTION OF GRIGNARD REAGENTS AND ORGANOLITHIUM COMPOUNDS	$Si - OH + SOCl_2 \xrightarrow{\text{Toluene}} Si - Cl + SO_2 + HCl$ a). $Si - Cl + BrMgR \rightarrow Si - R + MgClBr$ or b). $Si - Cl + Li - R \rightarrow Si - R + LiCl$	Si - C
a). TES SILANIZATION	a). $Si - OH$ $Si - OH \rightarrow$ $Si - O - Si - H$ (O) $Si - OH$ $Si - O - Si - H$ (O) $Si - O - Si - H$ (O)	a). Si - H monolayer
b). HYDROSILATION	b). $Si - H + CH_2 = CH - R \xrightarrow{\text{Catalyst}} Si - CH_2 - CH_2 - R$	b). Si - C

Figure 5 – Types of Reactions for Producing Bonded Phases.

The second step is the attachment of the organic moiety to the hydride intermediate. This is usually accomplished by passing a solution containing a terminal alkene and a suitable a catalyst such as hexacholoroplatinic acid through the capillary. This second step is referred to as hydrosilation.

$$- Si - H + R - CH{=}CH_2 \rightarrow^{cat} - Si\,CH_2 - CH_2 - R \qquad (6)$$

One particular benefit of this bonding method is attachment of the organic moiety to the surface via a stable Si – C linkage. In this method a significant number of silanols are converted to hydrides which lowers the

electroosmotic flow. This results in an increase in time for solute-bonded phase interactions and an enhancement of separation due to the differences in electrophoretic mobility of the charged species. These alterations to the fused silica wall create a surface that is more biocompatible than bare or unetched capillaries. The versatility, ruggedness and high resolving power of etched chemically modified capillaries for OTCEC has been demonstrated in a number of publications (28). A similar approach was used to evaluate them again but changing the surface matrix by adding inorganic salts during the etching process.

Chemical modifications of the capillaries are a step for better efficiencies in certain separation processes. But this leads to a diminished number of silanol groups on the inner surface of the capillary that affects the electroosmotic flow. When voltage is applied a weak electroosmotic flow will be developed, as not many free silnaol groups are present.

1.4 Electroosmotic Flow (EOF)

Electroosmotic flow is due to the electric double layer that develops at the silica solution interface. The silica surface has free silanol groups that deprotonate over a pH range of 3-7. Buffer cations associate with the negatively charged surface of the capillary. These cations move towards the cathode under the influence of the electric field. Since the cations are solvated they pull the bulk solvent along with them. Electroosmosis leads to a bulk solution flow that has a flat profile. The EOF developed is strong enough to

move all ions towards the cathode irrespective of their charge and mass. The strength of the EOF developed depends on the number of free silanol groups present on the surface. The greater the number of silanols present on the surface, the greater is the EOF developed. Hence in the bare capillary the EOF developed is generally higher when compared to modified capillaries where the silanol groups are either masked or modified by temporary coatings or permanent covalent modifications for the achievement of better separations.

EOF is significant because it affects the amount of time a solute resides in the capillary. If the EOF is low, sometimes it results in better solute wall / bonded group interactions leading to better resolution. If the EOF is very large the components of the mixture may not have adequate on-capillary time which may be better in some cases where there is a need to avoid solute-wall interactions. In some cases large EOF doesn't give better resolution because the solute species co-migrate, if the separation is based on the electrophoretic mobilities alone as it adds the same velocity component to all solutes regardless of their ionic status. Hence the separation efficiency and resolution of solutes are directly related to the direction and the magnitude of electroosmosis. Sometimes, the EOF can be reversed by changing the surface of the capillary. If the surface of the capillary is coated with positively charged ions then the anions of the buffer will congregate at the surface resulting in a negative double layer. This leads to flow towards the anode. It is important to have some idea of the magnitude of the EOF for better

understanding of the resolution process. Secondly, it is useful to know where neutral compounds migrate in the electropherogram obtained.

In one method EOF is measured by injecting a neutral marker and letting to travel through the capillary under the influence of applied voltage. It is detected and the EOF is calculated from the time taken by the neutral marker to travel along the length of the capillary. This is more applicable for a bare capillary as it has a greater number of silanols that generate higher EOF. Surface coatings and chemical modifications of the inner surface of the capillary reduce the number of silanols available for producing EOF. In such cases the EOF will be low and it will take a longer time for the neutral marker to reach the detector. In such cases the double peak method is employed where the neutral marker is driven by pressure instead of applied voltage (29). The first marker is introduced and voltage is applied which makes the marker move a certain distance. Then a second marker is introduced. Now the two markers are driven towards the detector by applying pressure. The EOF is calculated by the difference in the migration distance of the two markers.

The aim of this work is to evaluate and compare capillaries that were etched in the presence of different inorganic salts prior to chemical modification by a hydrophobic C18 moiety. The change in composition of the surface of the capillary after etching in the presence of five different inorganic salts was studied by the separation of small peptides. Capillaries etched in the presence of different inorganic salts and later modified by C18 are tested

along with comparing capillaries etched alone in the presence of salts, and a capillary etched in the absence of salts and later modified by C18. The EOF of each type of capillary was measured by the double peak method using the neutral marker DMSO. The bare capillary, capillaries etched only in the presence of salts and capillaries etched in the presence of salts and then C18 modified were evaluated by separating a mixture of enkephalins, natural opiates found in the central nervous system. Some capillaries were tested with a mixture of heterocyclic aromatic amines. In all cases, the separations were optimized by varying the pH over the range of 2-7 and using an organic modifier like methanol or acetonitrile in the mobile phase.

2. EXPERIMENTAL

A series of experiments were planned and performed based on the goals of this research work. They include the conditioning, etching and modification of the capillaries. Additional experiments were conducted to determine the EOF of the modified capillaries. All these experiments are described in detail under the experimental section. The chemicals required are listed in a tabular form (Table 1).

2.1. Reagents and Materials

Table 1: List of Chemicals Used in This Study Along With Their Chemical Abstract Service (CAS) Numbers

Name	Source	CAS Registry Number
β-alanine	Sigma	[107-95-9]
γ-amino butyric acid	Sigma	[56-12-2]
2-(N-morpholino) ethane sulfonic acid	Sigma	[4432-31-9]
Acetone	Fischer Scientific	[67-64-1]
Ammonium hydrogen difluoride	Aldrich Chemicals	[1341-49-7]
Chloroplatinic acid	Sigma-Aldrich Chemicals	[16941-12-1]
Citric acid	J.T. Baker Chemicals	[77-92-9]
Dimethyl Sulfoxide	EM Science	[67-68-5]
Dioxane	GFS Chemicals	[1764-74-4]
1-Octadecene	GFS Chemicals	[112-88-9]
Sodium Hydroxide	Fischer Scientific	[1370-73-2]

Name	Source	CAS Registry Number
Methanol	Spectrum Chemicals	[67-56-1]
Toluene	Fischer Scientific	[108-99-8]
Triethoxysilane (TES)	TCI America	[998-30-1]
Tris [hydroxymethyl] Amino methane	Sigma	[77-86-1]
Acetic acid glacial	EM Science	[64-19-7]
Lactic acid	J.T. Baker Chemicals	[50-21-5]
Histidine	Sigma	[4998-57-6]
Boric acid	J.T. Baker Chemicals	[10043-35-3]
2-(N-morpholino) propane sulfonic acid	Sigma	[68399-77-9]
Imidazole	Eastman Organic Chemicals	[288-32-4]
D-Ala^2, D-Leu^5 Enkephalin	Sigma	[94825-57-7]
Met^5 Enkephalin	Sigma	[58569-55-4]
D-Ala^2, D-Met^5 Enkephalin	Sigma	[100929-58-6]
Leu^5 Enkephalin	Sigma	[58822-25-6]

Name	Source	CAS Registry Number
2-Amino-3,4-dimethylimidazole [4,5f-] quinoline, MeIQ	Toronto Research Chemicals	[77094-11-2]
2-Amino-3,8-dimthyl-imidazole [4,5-f] quinoline, MeIQx	Toronto Research Chemicals	[77500-04-0]
2-Amino-9H-pyrido-[2,3-b] indole, AαC	Toronto Research Chemicals	unknown
2-Amino-1-methyl-6-phenyl-imidazole [4,5-b] pyridine, PhIP	Toronto research Chemicals	[105650-23-5]
2-Amino-3-methylimidazo-[4,5-f], IQ	Toronto Research Chemicals	[76180-96-6]

2.2 Instrumentation

The various instruments that were used for this research and how they were used in the present work are explained in depth in the following sections.

2.2.1 Capillaries

The capillary tubing used was 375 μm o.d. X 50 μm i.d. (Polymicro tetchnologies, Phoenix, AZ) made of fused silica. The capillary had an external coating of polyimide.

2.2.2 Gas Chromatography Oven

The etching and modification were carried out in a Hewlett-Packard Model 5890 gas chromatography oven. The GC apparatus was modified so that several capillaries can be placed at the inlet. Thick walled glass tubes were used as reservoirs for the etching and modification processes. These reservoirs were held in metal containers in the GC apparatus. The glass tubes had Swagelok fittings so that nitrogen gas could be used to force the reagents through the capillary inlet that was immersed in the reagent tube. The Swagelok fittings had small holes made through them so that the capillaries can be inserted tightly. Each Swagelok fitting has 1-5 holes. The particular reagent was placed into a plastic vial which in turn is placed into the glass reservoir. The capillaries were inserted through the holes of the Swagelok fittings and they were connected tightly to the glass tubes so that the inlet end of capillary that was through the Swagelok fitting was immersed in the reagent. The outlet of the capillary was placed in a collection vial.

2.2.3 CE Instrument

The etched and modified capillaries were tested using an Agilent (Waldbronn, Germany) 3D capillary electrophoresis system with a UV detector. Electrophoretic data were processed using HP Chemstation software. The applied voltage was +25 kV. The temperature was held at 25 ^{o}C. The hydrodynamic injection mode was used with 50 mbar of pressure applied for 3 sec. Direct detection was performed at 214 nm for the neutral

marker DMSO, 210 nm for the enkephalin mixture and 263 nm for the heterocyclic amine mixture.

2.3 Methods

Some of the experimental methods that were carried out in the present work like the synthesis of capillaries were based on the methods of Pesek and Matyska (30). Some were designed as needed. All the experimental methods are described step by step here in each subsection.

2.3.1 Etching of the Capillaries

The etching process was carried out following the method of Pesek and Matyska (31). The procedure described earlier involved connecting five 50 μm i.d. bare capillaries in the GC oven. The bare capillaries were inserted through the Swagelok fittings. The inlet plastic vial was filled with concentrated HCl. This vial was placed in the thick glass tube which in turn was placed in the metal containers of the GC oven. The fittings along with the capillaries are connected tightly to the glass tubes with the inlet end of the capillaries going through the fitting being immersed in the concentrated HCl reagent. A nitrogen gas pressure of 35 psi was applied to fill the capillaries with HCl. The outlets of the capillaries were checked to determine if HCl flowed into the capillaries. The capillaries were coiled and covered in aluminum foil and heated overnight at 80 ^{o}C. The capillaries were washed successively by flushing them with deionized water, acetone and methanol for

approximately 15 min with each solvent. Finally, the capillaries were dried by passing nitrogen gas through the capillaries at 40 psi.

2.3.2 Preparation of Saturated Solution of the Inorganic Salt and the Etching Solvent

Five different salts, cupric chloride, chromium chloride, calcium nitrate, sodium nitrate and ammonium carbonate, were selected to be evaluated in the etching process. The choice of inorganic salts was made based on their solubility in methanol, the solvent used for making the etching reagent. Initially the weight of salt required to make a saturated solution in methanol was determined by taking 5 ml of methanol and adding the salt slowly with constant stirring. The process was continued until no more salt dissolved. Then the remaining salt was weighed and the weight was subtracted from the initial weight of the salt. Then the amount of inorganic salt required was calculated to approximately make a saturated solution of the salt along with the etching reagent.

A 5% (w/v) saturated solution of ammonium hydrogen difluoride was prepared in methanol. A 15 % (w/v) saturated solution of cupric chloride was made using the saturated solution of ammonium hydrogen difluoride. This etching solution was placed in a plastic vial and five of the previously conditioned capillaries were filled with this solution by applying 40 psi nitrogen gas pressure. The capillaries were left for an hour filled with the etching solution. The ends of the capillaries were sealed by covering them in

aluminum foil and heated in the oven for four hours at 300 °C in the presence of nitrogen. After removal from the oven, the capillaries were checked under a light microscope to detect any blockages. Then they were flushed with methanol for one hour to remove any excess etching solution and later they were dried using nitrogen gas.

2.3.3 Formation of the Hydride Layer

The etched capillaries were filled with 0.1 M NaOH (pH ~ 12) and left for 20 hours. The capillaries were rinsed with deionized water until the effluent was approximately pH 7 (checked with pH paper) and then with 0.1 M HCl for four hours. They were again flushed with de-ionized water until the effluent was neutral (checked with pH paper). The capillaries were rinsed with acetone for 15 min and heated at 100 °C for 24 hours in the presence of nitrogen gas. This process is the preconditioning of the capillaries i.e., making them ready for the formation of the hydride layer.

For silanization of the capillaries a 1 M TES solution was made in 6.4 mL of dioxane and 100 μl of 2.3 M HCl in the presence of the inert gas argon. The capillaries were filled with the TES solution at 40 psi nitrogen gas pressure and then covered in a foil and heated for 90 min at 90 °C. This process results in the formation of a hydride layer in the capillary. Then the capillaries were washed with dioxane for two hours. Next they were washed with toluene for an additional two hours. The capillaries later were dried with

nitrogen gas for one hour before attaching the appropriate organic moiety. The step by step process of etching and modification is shown in the Figure 6.

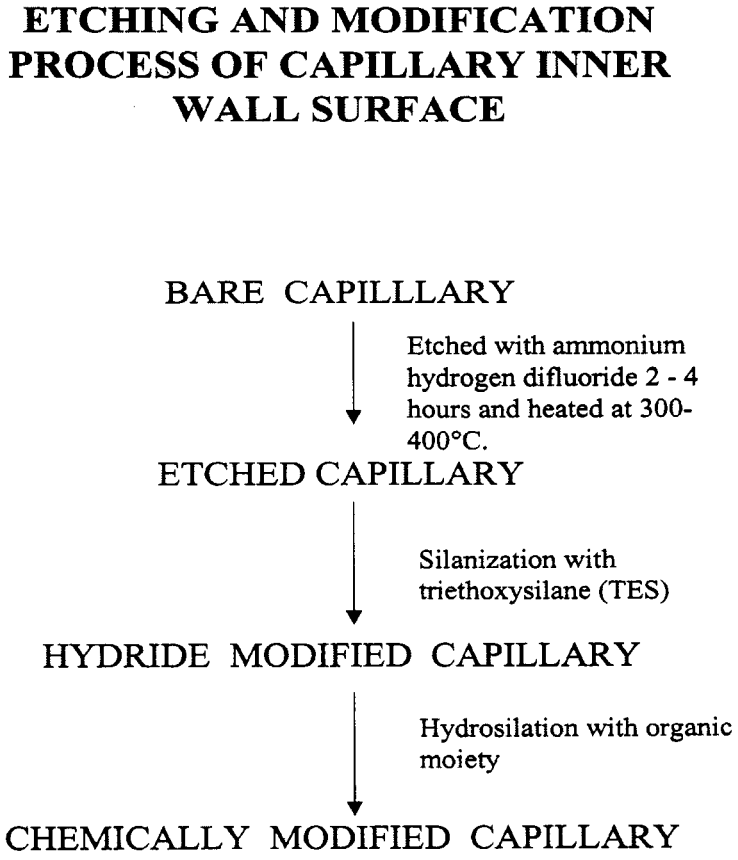


Figure 6 – General Scheme of Capillary Modification Process

2.3.4 Activation of the Bonding Material

The above etched capillaries were chemically modified by attaching 1-octadecene as the organic moiety. Before attaching the alkyl chain, it has to be activated. First 2 mL of pure 1 - octadecene was mixed with 70 μL of 10

mM Spier's catalyst (hexachloroplatinic acid in 2-propanol) and placed along with a magnetic stir bar in a three neck round bottom flask. The flask was placed on a heating mantle that was connected to a thermostat. The whole assembly, that is, the three neck flask along with the heating mantle was placed on a magnetic stirrer. A thermometer was inserted through one of the necks of the flask. A West condenser was inserted into the middle neck of the flask and cooling water was circulated through it. The other neck was covered with a stopper. The contents were refluxed for one hour at 70 °C.

2.3.5 Attachment of the C-18 Moiety

The etched capillaries that were hydride modified were filled with activated 1-octadecene for 90 hours at 100 °C. Upon completion the capillaries were flushed with toluene and methanol for one hour with each solvent and dried with nitrogen gas at 100 °C.

Similarly, a set of five capillaries for the other inorganic salts, chromium chloride, calcium nitrate, sodium nitrate and ammonium carbonate were etched and chemically modified by forming the hydride layer and later attaching the C-18 moiety as described above.

2.4 Preparation of Buffers

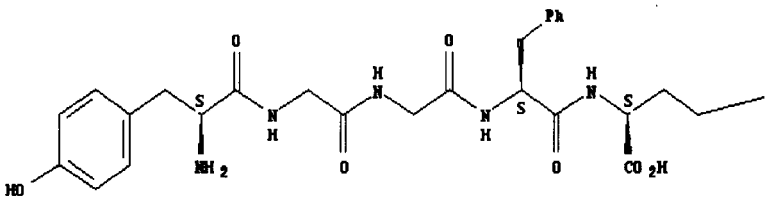
The buffers used in the study were: pH = 2.14, 30 mM phosphate and 19 mM Tris; pH = 3.00 30 mM citric acid and 25 mM β-alanine; pH = 3.70, 136 mM β-alanine and 30 mM lactic acid; pH = 4.41, 30 mM acetic acid and

37.5 mM γ-amino butyric acid; pH = 6.00, 30 mM 2-(N- morpholino) ethanesulfonic acid and 21 mM histidine; pH = 7.06, 30 mM 2-(N-morpholino) propanesulfonic acid and 21.5 mM imidazole; pH = 8.14, 10 mM Tris and 15 mM boric acid. Deionized water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA) and was filtered through a 0.20 μm Nylon 66 membrane filter (Altech Assoc., Deerfield, IL). The buffers were diluted with deionized water in 1:10 ratios with one part of buffer and 10 parts of deionized water. Helium gas was sparged through the buffers to degas the solutions which removes most of the air from the solution. Sparging was done by using a suitable length of PTFE tubing connected to the outlet of a regulator on a helium cylinder. A sparging frit was connected to the other end of the tubing which is immersed in the buffer. Diluted buffer with water in the ratio of 1:10 were made and stored. Buffers with 50% organic modifier were also made in separate bottles and stored in the refrigerator. Methanol and acetonitrile were the two organic modifiers used in this study.

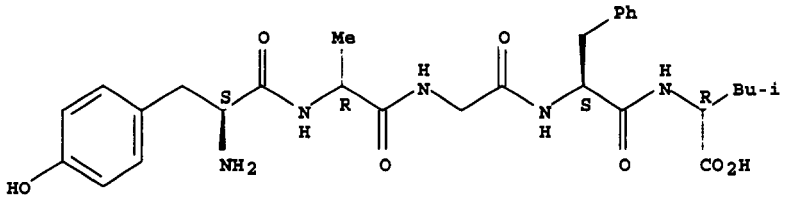
2.5 Preparation of the Samples

All the solutes were water-soluble. 1 mg of each compound was dissolved in 1 mL of deionized water (0.1%) and stored in vials in a freezer. Similarly, a mixture of all three enkephalins was prepared at a concentration of 0.1%. Heterocyclic aromatic amines were also water soluble. 1 mg of each compound was dissolved in 1 mL of water and a mixture at a concentration of

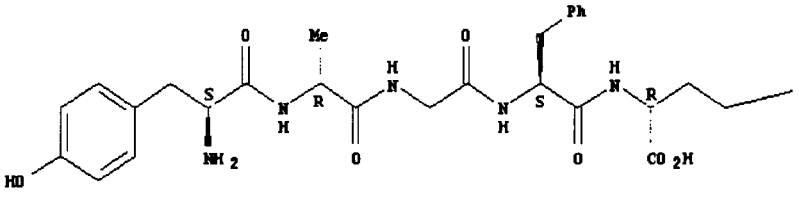
0.1% was made and stored in a refrigerator. Figure 7 shows the structures of the enkephalins. The heterocyclic aromatic amines are shown in the Figure 8.



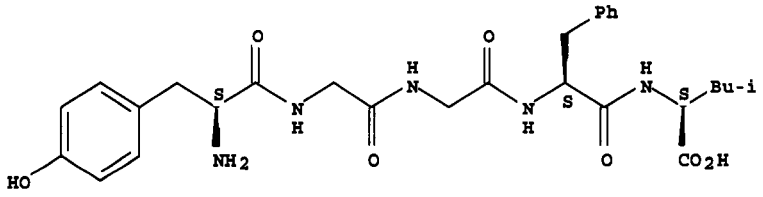
[Met^5] Enkephalin



[D- Ala^2, D- Leu^5]- Enkephalin



[D-Ala^2, D-Met^5]- Enkephalin



[Leu^5] Enkephalin

Figure 7 - Chemical Structures and Names of Enkephalins

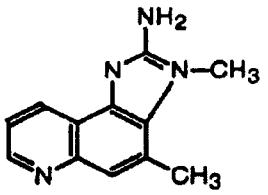
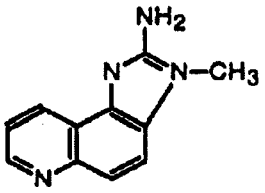
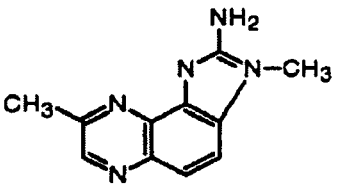
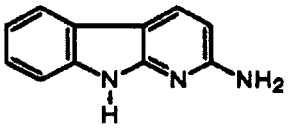
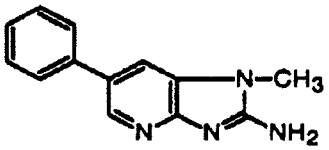
	Amine	Structure
MeIQ	2-Amino-3,4-dimethyl-imidazo[4,5-*f*]quinoline	
IQ	2-Amino-3-methylimidazo-[4,5-*f*]quinoline	
MeIQx	2-Amino-3,8-dimethyl-imidazo[4,5-*f*]quinoxaline	
AαC	2-Amino-9*H*-pyrido-[2,3-*b*]indole	
PhIP	2-Amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine	

Figure 8 – Chemical Structures and Names of Heterocyclic Aromatic Amines

2.6 Measurement of EOF

The EOF of the bare, etched and etched chemically modified capillaries was measured by the double peak method. For a bare capillary the EOF was measured by both the traditional and double peak method. For all other capillaries only the double peak method was employed. A 1% dimethyl

sulfoxide (DMSO) solution was used as the neutral marker to measure the EOF. About 1 mL of DMSO was dissolved in 100 mL of water and stored at room temperature.

2.6.1 Traditional Method

The neutral marker was injected at 50 mbar of pressure for 3 sec and was detected at 214 nm when it passed the detector. Because the neutral marker migrates at the rate of the EOF, the velocity of the neutral marker is used to calculate the EOF.

2.6.2 Double Peak Method

In the case of modified capillaries, the EOF was measured by the double peak method. The neutral marker was injected as usual and a 10 kV voltage was applied for a specific period of time (varied in each capillary) and then a second injection was made. These two markers were pushed along with a plug of electrolyte in between them, to the opposite end of the capillary by applying 50 mbar pressure without any voltage. The EOF was calculated using Eq. 7 and plots of EOF vs. pH are shown in Figures 9-15.

$$\mu_{eo} = Ll / Vt_v \times (1-t_1/t_2) \qquad \text{Eq 7}$$

Where: L = total length of the capillary; l = effective length of the capillary; V = applied voltage; t_v = time for which voltage was applied; t_1 and t_2 = migration times of the two markers.

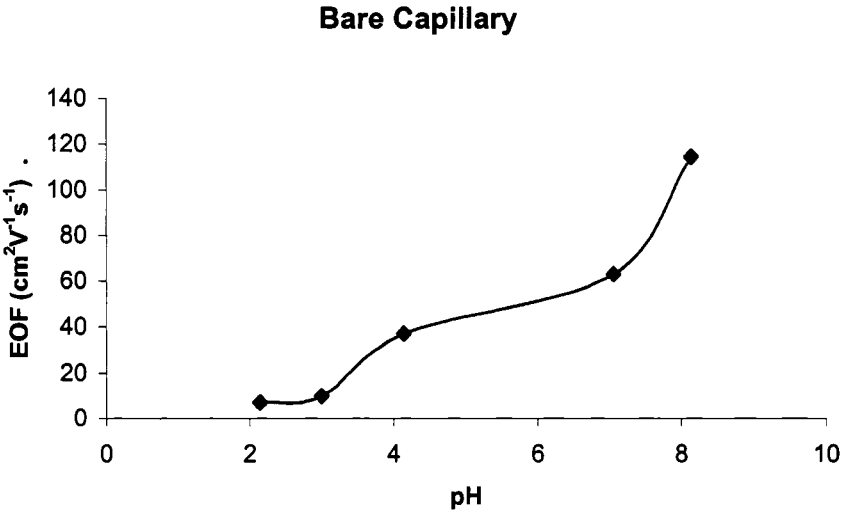


Figure 9 - Graph of EOF vs. pH of Bare Capillary

Electroosmotic flow as a function of pH in a 50 μm I.D. bare capillary Marker is dimethyl sulfoxide (DMSO) with detection at 214 nm. Each point is an average of three measurements. EOF values multiplied by 10^5.

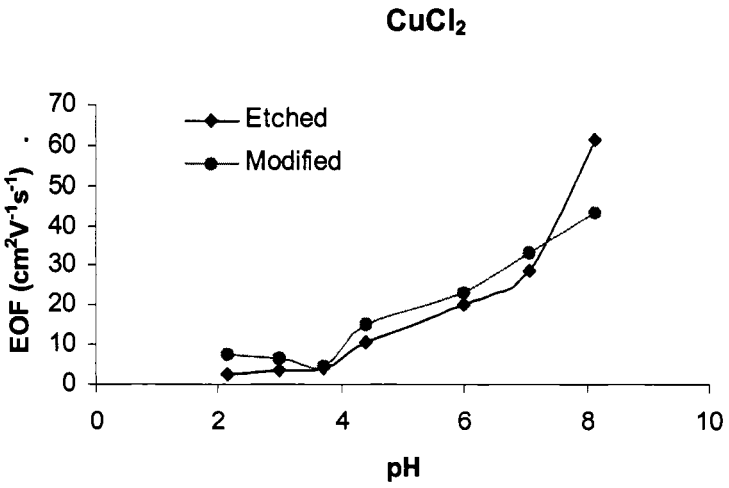


Figure 10 – Graph of EOF vs. pH of Capillaries Etched with $CuCl_2$, and C18 Modified.

Electroosmotic flow as a function of pH in the 50 μm I.D. etched with cupric chloride, etched with cupric chloride and C18 modified capillaries. Marker is dimethyl sulfoxide (DMSO) with detection at 214 nm. Each point is an average of three measurements. EOF values multiplied by 10^5.

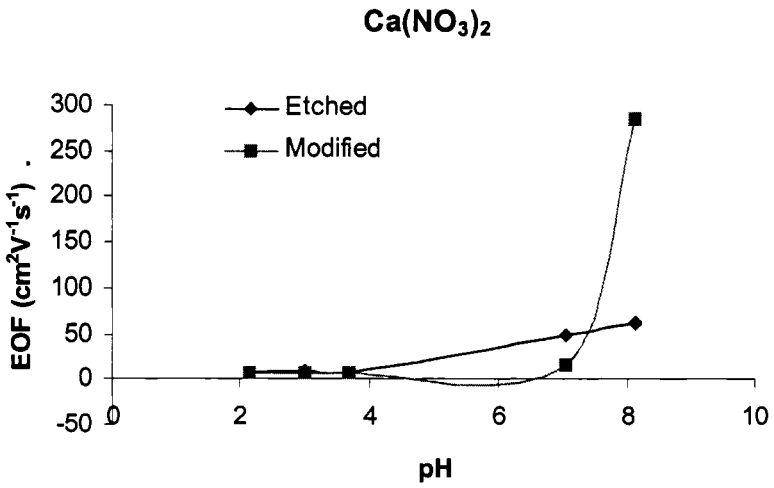


Figure 11 – Graph of EOF vs. pH of Capillaries Etched with $Ca(NO_3)_2$ and C18 Modified.

Electroosmotic flow as a function of pH in the 50 μm I.D. etched with calcium nitrate, etched with calcium nitrate and C18 modified capillaries. Marker is dimethyl sulfoxide (DMSO) with detection at 214 nm. Each point is an average of three measurements. EOF values multiplied by 10^5.

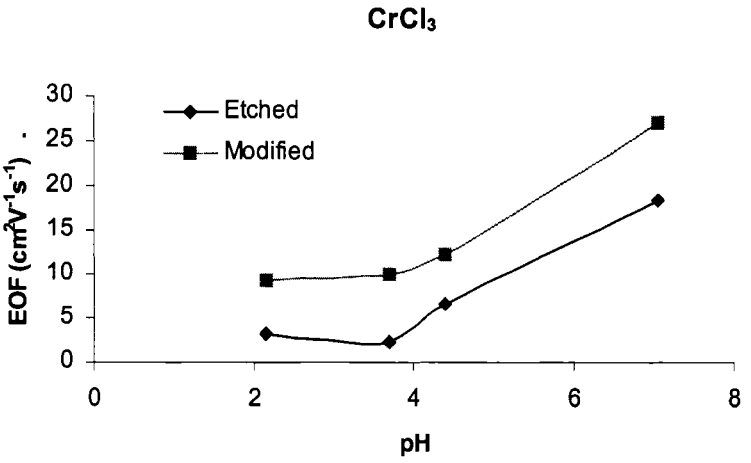


Figure 12 - Graph of EOF vs. pH of Capillaries Etched with $CrCl_3$ and C18 Modified

Electroosmotic flow as a function of pH in the 50 μm I.D. etched with chromium chloride, etched with chromium chloride and C18 modified capillaries. Marker is dimethyl sulfoxide (DMSO) with detection at 214 nm. Each point is an average of three measurements. EOF values multiplied 10^5.

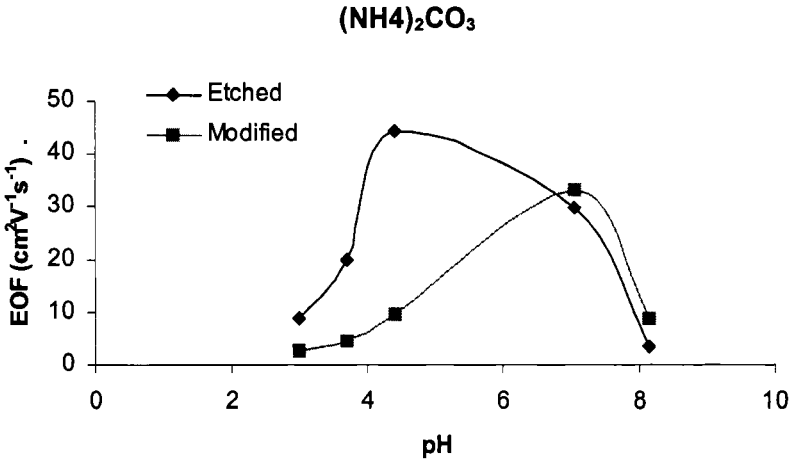


Figure 13 - Graph of EOF vs. pH of Capillaries Etched with $(NH_4)_2CO_3$ and C18 Modified.

Electroosmotic flow as a function of pH in the 50 μm I.D. etched with ammonium carbonate, etched with ammonium carbonate and C18 modified capillaries. Marker is dimethyl sulfoxide (DMSO) with detection at 214 nm. Each point is an average of three measurements. EOF values multiplied by 10^5.

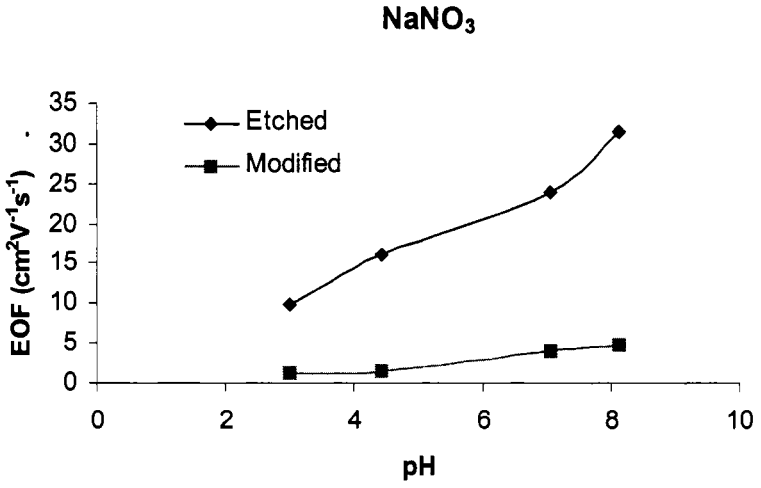


Figure 14 – Graph of EOF vs. pH of Capillaries Etched with $NaNO_3$ and C18 Modified.

Electroosmotic flow as a function of pH in the 50 μm I.D. etched with sodium nitrate, etched with sodium nitrate and C18 modified capillaries. Marker is dimethyl sulfoxide (DMSO) with detection at 214 nm. Each point is an average of three measurements. EOF values multiplied by 10^5.

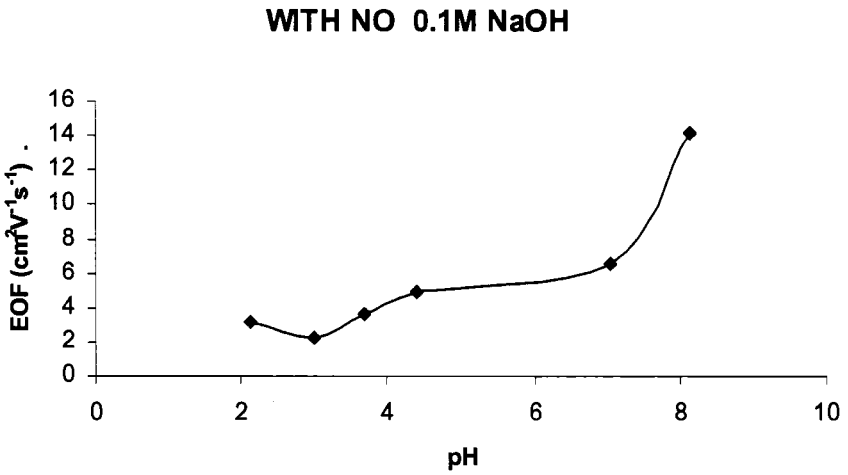


Figure 15 – Graph of EOF vs. pH of Capillary Etched with $CuCl_2$ and C18 Modified Without Treating with 0.1M NaOH.

Electroosmotic flow as a function of pH in the 50 μm I.D. etched with cupric chloride and C18 modified not treated with 0.1M NaOH capillary. Marker is dimethyl sulfoxide (DMSO) with detection at 214 nm. Each point is an average of three measurements. EOF values multiplied by 10^5.

2.7 Separation of Samples

Using each capillary the sample mixture was tested with all running buffers at a voltage of 25 kV. The sample mixtures were separated using a total of 21 buffers. The injection duration was 3 secs. Prior to running the sample mixture, the capillary was preconditioned by sodium hydroxide in the case of the bare capillary, deionized water and the running buffer each for five minutes. For the etched and etched chemically modified capillaries methanol was used instead of sodium hydroxide.

3. RESULTS AND DISCUSSION

3.1 Etching and Modification of the Capillaries

There are various methods for the modification of fused silica capillaries. The fused silica capillary consists of silanol groups in different states. These silanol groups are weak acidic sites that tend to deprotanate over a range of pH 3 – 7. During the course of separation of compounds like proteins and peptides, this is a problem as the cationic sites of the proteins adhere to the negative silanol groups making the separation nonreproducible and even impossible in some cases (32). For this reason it can be beneficial to modify the inner surface of the capillary by bonding an appropriate organic moiety that acts as the stationary phase, to prevent the adsorption of solutes. But the main drawback is the low phase ratio of the bonded material due to the small surface area that is available on the wall for the stationary phase. Hence the amount of the stationary phase present will be insignificant. To mitigate this drawback and to increase the surface area the capillaries are etched.

In the process of etching when the capillaries are treated with a saturated solution of 5% ammonium hydrogen difluoride followed by heating at high temperatures, strong protonic sites are created on the silica surface by the partial replacement of –OH groups by –F and the resulting surface exhibits high catalytic activity (33). The electronegative fluorine atom causes an electronic shift in the neighboring atoms, resulting in the weakening of the

hydroxyl bond in the silanol group and an increase in the surface acidity. These acidic groups are of the Bronsted type (Figure 16) (33). The capillary surface now consists of Si-OH and Si-F groups. Due to these site-specific

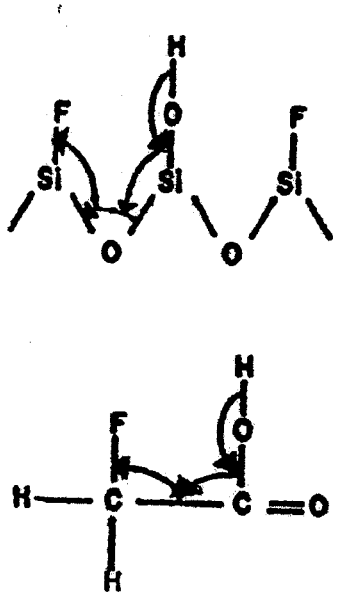


Figure 16 – Bronsted Type Acidic Groups

The electronegative fluorine atom causes an electron shift in the neighboring atoms resulting in the weakening of hydroxyl bonds.

reactions the original surface of the fused capillaries first dissolves non-uniformly in the ammonium hydrogen difluoride solution and then precipitates back onto the wall in large particles. It also produces radial extensions of several microns in length from the surface. This reduces the

distance the solute must travel in order to interact with the stationary phase. The capillary surface area increases up to 1000-fold, thus opening up sites for the attachment of the stationary phase material (30). After the reaction with the etching agent, the heating procedure (300 °C for four hours) leads to the formation of a more uniform and a better-defined surface. The inner etched surface of the capillary with radial extensions has been examined previously by atomic force microscopy. These pictures clearly revealed the surface morphology of the capillary. The new surface resembles sand dunes with a rugged texture (34). The mechanical stability of these surface structures has been studied by vibration of the capillaries for 30 minutes (35). These studies found that the surfaces produced under the above conditions do not undergo any damage and can withstand the mechanical stress from vibration.

Prior to etching, the etching agent was saturated with the inorganic salt of interest. These inorganic salts were also incorporated in the surface. When the capillary dissolves in the etching agent during the etching process and re-precipitates, it is thought that the ions of the corresponding salt present in the solution also precipitate onto the surface, thus changing the surface matrix. In that case the inner surface matrix has inorganic ions of the salt used in that particular capillary like cupric and chloride, sodium and nitrate, calcium and nitrate, chromium and chloride and ammonium and carbonate ions along with ammonium and fluoride ions of the etching agent. The effect of these salts on the surface morphology was studied first by measuring the EOF of each

etched capillary and later the EOF of capillaries that were etched and subsequently chemically modified. The effect was also monitored by the separation of a mixture of enkephalins and the separation of a mixture of heterocyclic aromatic amines, which are basic compounds.

After etching there is a two-step modification of the capillary. First the etched surface is treated with TES, which removes the silanol groups that were exposed by the etching agent, and a monolayer of silica hydride is formed. This reaction process is called silanization.

The next step, the attachment of the hydrophobic alkyl moiety is called hydrosilation. This process requires the presence of a catalyst such as a metal complex. The catalyst usually consists of halide, or a phosphine complex of transition metals such as platinum, palladium etc. In this study a 2-propanol solution of hexacholroplatinic acid, called Spier's catalyst was employed. In general 10^{-7} to 10^{-4} mol of platinum / mol of silicon hydride is sufficient for an effective hydrosilation (26). In the case of simple liquid olefins such as C-18 no additional solvent is required. The reaction is carried out under dry conditions at 60 °C. Figure 17 shows the structure of the organic moiety before bonding. There have been evaluations of similar modified capillaries where the successful separation of several types of proteins and peptides show that a large number of the Si-H groups have been converted to the C-18 group (36, 37).

Figure 17 – Structure of 1 - Octadecene (C-18).

3.2 EOF Measurement

Chemical or physical modifications of capillaries are normally accompanied by a drastic reduction of electroosmotic flow. Since EOF has a strong impact on electrophoretic separations its measurement is important for a thorough characterization of the separation process and the electrophoretic system. Usually EOF measurements are done by injecting a neutral marker. The neutral marker has no charge. It moves along with the velocity of the electroosmotic flow. Thus it travels through the capillary towards the detector with little or no influence of the surface of the capillary, or the buffer ions of the running solvent used. So the calculated velocity of the neutral marker is equal to the EOF of a particular capillary. This traditional method works well for the bare capillary where there are many silanol groups. The presence of more silanol groups results in a greater EOF; hence the neutral marker travels to the other end of the capillary in a reasonable period of time. But in the case of modified capillaries where many of the silanol groups are made inactive by the attachment of the organic groups, there is insufficient EOF to move the neutral marker in a reasonable period of time. This creates a problem to measure the EOF, just based on the velocity of neutral marker by itself as it

takes a much longer period of time. In such situations the double peak method is employed for the measurement of EOF.

The double peak method is an adaptation of the traditional neutral marker approach. It is a fast pressure-driven mobilization of the neutral marker for accelerated electroosmosis measurements in chemically modified capillaries. The basic idea of this method is to speed up the electroosmotic velocity determination by measuring only a short fraction Δl of the effective length l over a short interval of time t_v. In contrast to the conventional method, the double peak method involves two marker injections where voltage is applied for a certain period in between the first and second injections of the marker.

A neutral marker is injected first. After the first injection a high voltage (V) is applied for a specific period of time (t_v). This creates a measurable amount of EOF which makes the neutral marker travel a certain distance. Then the voltage is stopped and a second injection is made. Now the two neutral markers are at a definite distance apart with a plug of electrolyte sandwiched between them. Then they are forced out to the detector end of the capillary by applying a specific amount of pressure. This causes the neutral markers along with the plug of sandwiched electrolyte to move rapidly towards the detector where they are detected in a reasonable period of time. The two marker injections are merely used to establish the ends of the electrolyte plug moved by the electric field. The EOF is

calculated by determining the difference in the migration times of the two neutral markers. The process of injecting the markers and applying pressure is shown in the steps in Figure 18.

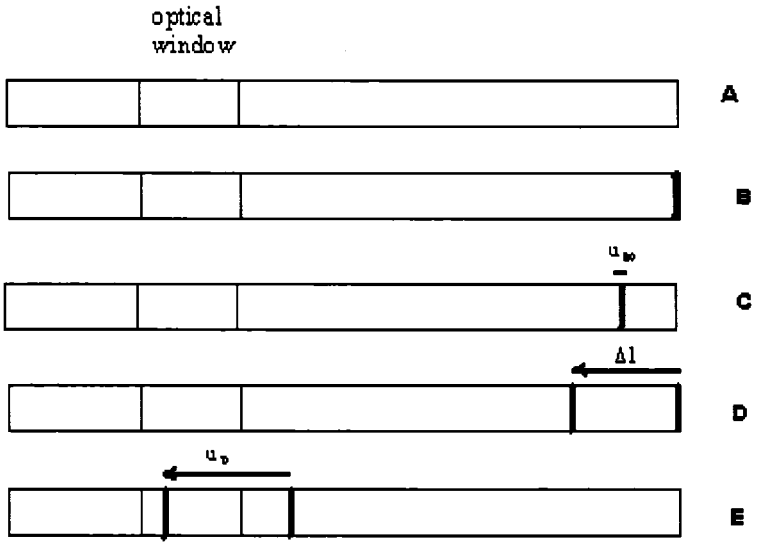


Figure 18 – Sequence of Steps in Double Peak Method

(A) The capillary tube is initially rinsed with electrolyte. (B) A marker injection is made. (C) Electric field is applied for a fixed time (t_v) to electroosmotically move the marker band. At the end of this period the marker band along with a plug of electrolyte have traveled a distance Δl. (D) A second injection is used to indicate the end of the electrolyte plug. (E) Upon application of a pressure differential between inlet and outlet ends (positive pressure or vacuum) the marker-sandwiched electrolyte plug reaches the detection window and the traveling times for the two marker bands are recorded in the usual manner (29).

3.2.1 Derivation:

During the application of the electric field (Step C in Figure 18), the electroosmotic velocity of the electrolyte $u_{eo,}$ is approximately equal to

$$u_{eo} \approx \Delta l / \Delta t \qquad \text{Eq (8)}$$

where Δl is the distance traveled by the electrolyte and Δt is the travel time, i.e., the period during which the voltage is applied (t_v). Assuming the flow velocity, u_p is maintained constant during the pressure-driven mobilization (Step E in Figure18) the second marker travels over the entire effective length, l while the first one does so over a shorter distance $l - \Delta l$.

Hence,

$$u_p = l / t_2 = l - \Delta l / t_1 \qquad \text{Eq (9)}$$

where t_1 and t_2 are the pressure-driven mobilizations for the first and second times for the first and second marker bands. Solving for Δl one obtains

$$\Delta l = (1 - t_1 / t_2) \qquad \text{Eq (10)}$$

Thus, u_{eo} can be expressed as

$$u_{eo} = l / t_v(1 - t_1 / t_2) \qquad \text{Eq (11)}$$

Finally, from the definition of electroosmotic mobility, $\mu_{eo} = \{u_{eo} (L/V)\}$, L being the total length of the capillary, V the voltage applied, the formula to calculate the electroosmotic mobility can be obtained as

$$\mu_{eo} = Ll / Vt_v (1 - t_1 / t_2) \qquad \text{Eq (12)}$$

Previously, it has been determined that this method of measuring EOF is equivalent to the traditional method (27). The EOF was calculated for all capillaries used in this study: five capillaries each etched with a different salt, and later five chemically modified capillaries with the respective salt. A total of twelve capillaries including the bare capillary were tested using this double peak method.

3.3 Effect of pH on the EOF

Table 2: EOF Observations in All Capillaries

Salt Used	EOF Observations		
	Cathodic / Anodic	Increase/ Decrease	Special Properties
Cupric Chloride	Cathodic flow	Increased with an increase in pH.	Dip at pH 3.7. Increased sharply in modified capillary at pH 8.14.
Chromium Chloride	Cathodic flow	Constant increase with an increase in pH.	Etched has lower EOF values than its modified capillary.
Ammonium Carbonate	Cathodic flow	Increased initially and then decreased.	EOF highest around pH 3-4 and then dropped sharply.
Sodium Nitrate	Cathodic flow	Gradually increased	Etched has much higher values than its modified capillary.
Cupric Chloride but without 0.1M NaOH	Cathodic flow	Increased with pH increase.	Lower EOF values when compared to modified $CuCl_2$ and treated with 0.1M NaOH.

Salt Used	EOF Observations		
	Cathodic / Anodic	Increase / Decrease	Special Properties
Calcium Nitrate	Cathodic flow	Increased slowly with increasing pH	Very low EOF values for both etched and modified. EOF increased sharply at pH 8 for modified capillary.

Generally, as the pH of the buffer used increases the number of silanol groups (Si-OH) that deprotonate increase resulting in a higher EOF. As mentioned previously, the silanol groups deprotonate above pH 3. So as the pH increases a greater number of silanols lose protons and hence the electroosmotic mobility will be greater. This study included the following buffers: pH 2.14, pH 3.00, pH 3.70, pH 4.41, pH 6.00, pH 7.06 and pH 8.14. Electroosmotic mobility was calculated at each pH for all capillaries. Then a plot of EOF vs. pH was generated for the bare capillary, capillaries etched with salts and the capillaries etched with salts and then chemically modified. These plots of EOF versus pH for all capillaries indicate that the EOF increased with an increase in the pH (Figures 9-15). The EOF generated in the bare capillary is greater when compared to that generated in capillaries etched with salts and capillaries etched with salts and chemically modified as

expected. The bare capillary has a greater number of silanol groups when compared to the just etched and etched, chemically modified capillaries. As the pH increases more of the Si-OH groups lose protons leading to a higher EOF. Hence the EOF is considerably greater when compared to the chemically modified capillaries.

In chemically modified capillaries many of the Si-H moieties formed after silanization are reacted to covalently bond the organic group C18. There are few Si-OH groups to deprotonate. These Si-OH groups will lose protons as the pH exceeds 3. Hence the EOF generated with the minimum silanol groups present will be much less when compared to the purely etched and bare capillaries. Moreover, in almost all of the previous work done by Pesek and coworkers, it was observed that there was a negative EOF in etched chemically modified capillaries (38). It was proved by electron spectroscopic chemical analyses (ESCA) that during the etching process when the capillary dissolves and precipitates back, the ammonium ions that were present in the etching agent get incorporated in the surface matrix. Now the matrix has positive ions; hence at low pH the matrix is positively charged. The negative ions from the buffer form a double layer thereby reversing the direction of EOF resulting in a small anodic flow. Hence, a plot of EOF vs. pH shows a negative value (Figure 19). But as the pH is increased the positive ammonium ions are deprotonated and at the same time more silanol groups are deprotonated thereby creating an EOF that moves towards the cathode.

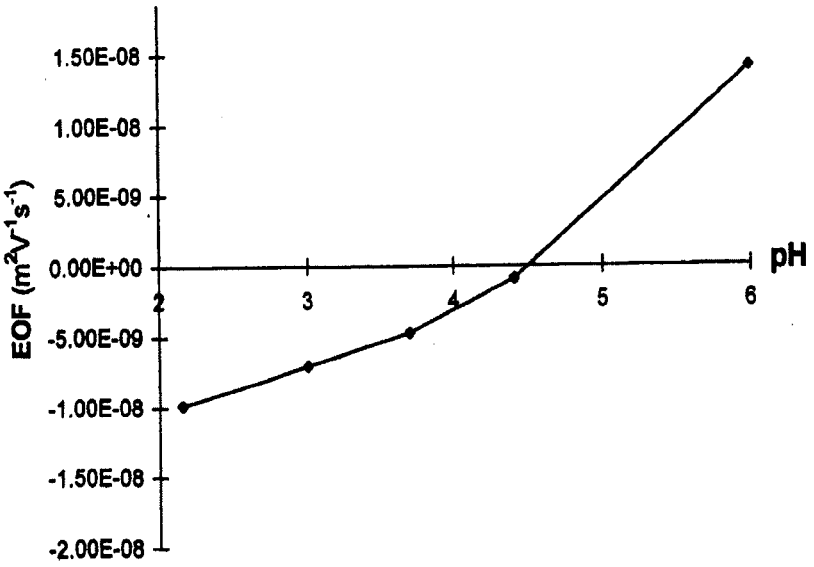


Figure 19 – Graph of EOF vs. pH of Etched and C18 Modified Capillary.

Electroosmotic flow as a function of pH in a 20 μm I.D etched C18 modified capillary. Marker is dimethyl sulfoxide (DMSO) with detection at 211 nm. Each point is an average of three measurements (38).

The presence of the salts in the surface matrix has shown some variations. Both types of capillaries, i.e., capillaries etched alone in the presence of salts and the capillaries that were etched in the presence of salts and subsequently chemically modified, have exhibited a different behavior. All capillaries that have additional salts in their surface matrix have generated a positive EOF instead of negative EOF. One possible explanation could be that the inorganic ions of the respective salt incorporate along with ammonium ions and the fluoride ions. As the inorganic ions also compete with ammonium ions to get into the matrix, fewer ammonium ions may have

been included in the matrix. The smaller number of ammonium ions are not sufficient to reverse the flow of EOF.

Some differences are found in the EOF values between chloride and nitrate salts used. The magnitude of EOF generated at a pH above 4.41 is smaller in the capillaries that were etched in the presence of calcium nitrate and sodium nitrate salts when compared to the capillaries that have cupric chloride and chromium chloride. It is possible that the presence of chloride ions is imparting more negative charge to the inner surface than the presence of the nitrate ions.

The other reason could be that the presence of nitrate salts is opening up more sites for bonding of the organic moiety so most of the Si-OH groups that remain are well shielded with the C18 group resulting in a low EOF. Finally, this can also be attributed to the amount of the salt present in the etching agent. That is the nitrate salts were much less soluble in the etching solution than the chloride salts. The two chloride salts were readily soluble in the etching solution. So, a greater number of ions might have been in the etching solution leading to fewer ammonium ions included in the surface matrix. Since the nitrate salts are less soluble the presence of fewer ions could lead to the incorporation of more ammonium ions in the matrix. The presence of a few more ammonium ions is not sufficient to reverse the EOF but enough to diminish the amount of cathodic EOF generated.

The etched and etched chemically modified capillaries with $(NH4)_2CO_3$ salt in the etching solution exhibited an unusual behavior. These capillaries also exhibited a positive cathodic flow. If the presence of ammonium ions from the etching agent reverses the flow, then the presence of ammonium carbonate salt should show a similar anodic flow. But that is not the case. Instead the EOF rose sharply from pH 2.14 to 7.06 and thereafter it dropped drastically (Figure 13). This could be attributed to the presence of carbonate ions in the surface matrix when compared to the number of ammonium ions. Or it could be that the ammonium ions may be deeper inside the surface matrix and carbonate ions are superficially closer to the surface in the matrix. Because carbonate ions are negatively charged a positive EOF is developed moving towards the cathode. This once again confirms that the presence of the inorganic salts during the etching process has definitely brought some change in the surface matrix leading to this unusual behavior of the capillaries.

In addition to the regular capillary etched with cupric chloride in the etching solution, one additional capillary was etched with the same salt in the etching reagent. Before the formation of hydride layer in the process of chemical bonding of an organic moiety, the capillaries were usually treated with 0.1 M NaOH solution for 20 hours. This is done to increase the number of silanol groups for silanization. This step was omitted in this one additional capillary to find out whether there would be any difference. Then the

capillary was chemically modified by bonding the C18 group. The plot of EOF vs. pH for this capillary revealed a slightly different pattern (Figure 15). The first noticeable point is that this capillary did not show any anodic flow. This could be due to the presence of more cupric and chloride ions when compared to the ammonium ions which might have changed the surface matrix leading to a cathodic flow. The second point was that the EOF values of the etched chemically modified capillary with cupric chloride salt not treated with 0.1 M NaOH solution before silanization are much lower when compared to the regular etched chemically modified capillary with cupric chloride salt. This indicates that fewer silanol groups are present in the capillary that was not treated with 0.1 M NaOH.

During the etching process the number of silanol groups especially free groups are lost because of the dissolution and precipitation of the capillary inner surface. But it is thought that the treatment with 0.1 M NaOH solution exposes free silanol groups that are later converted to hydrides by the silanization step. When this treatment with 0.1 M NaOH solution was not done, creation of the new silanol groups might not have occurred. Furthermore during the TES reaction, the silanization process removes an unknown number of silanol groups. All these factors might have contributed to the EOF properties consistent with the etched chemically modified capillaries with cupric chloride not treated with 0.1 M NaOH solution.

The EOF values of every capillary etched with each salt at all pH values (2.14, 3.00, 3.7, 4.41, 6.00 7.06 and 8.14) are lower when compared to the respective capillaries etched with same salts and later chemically modified though there are some variations at some pH values. This could be due to the fact that during the etching process many free silanol groups are lost on the inner surface of the capillary and hence lowers the electroosmotic flow. But treatment with 0.1 M NaOH solution after the etching process and before chemical modification creates some silanol groups though some are lost in the silanization process. Hence the capillaries etched with salts and later chemically modified have higher EOF values than their respective etched capillaries.

But the capillaries etched and chemically modified with sodium nitrate and ammonium carbonate in the respective etching solutions differed from this pattern. The EOF values of the capillaries etched with sodium nitrate and ammonium carbonate are greater than the etched C18 modified capillaries with the same salts (Figures 13 and 14). This raises the question whether the presence of the inorganic salts in the matrix affects only the surface matrix or do they have some affect on the bonding of the C18 moiety to the Si-H groups. The presence of the salts (ammonium carbonate and sodium nitrate) might have resulted in the loss of fewer silanol groups during the etching process. So the EOF developed is higher. Or they might have helped to increase the bonding of the Si-H groups to the C18 moiety during the

chemical modification. So the EOF developed in the etched chemically modified with the two salts $NaNO_3$ and $(NH_4)_2CO_3$ is lower. And the presence of other salts like cupric chloride, calcium nitrate and chromium chloride might have hindered the bonding of the organic C18 group to some extent. The EOF developed is greater in these etched and chemically modified capillaries with the above mentioned salts (cupric chloride, calcium nitrate and chromium chloride).

3.4 Test Solutes

Enkephalins are endogenous neuropeptides discovered by Hughes in 1975 (29). They exhibit a specific recognition with opiod receptors and play an important role in mediating mammalian stress and pain. They also have a vital role in numerous mammalian states and behaviors such as eating, tolerance, activity, learning, mental illness etc (30). Enkephalins are short penta peptides and have the same back-bone, Tyr-Gly. They are all structurally similar with a change in one or two amino acids in the sequence. Four different enkephalins used in this study are shown in the Table 3 along with their sequences. Due to the shortage of sample a few of the capillaries are tested only with three or two of the enkephalins.

Table 3: List of Enkephalins and Their Sequences

Peptide Name	Sequence
[Met^5] Enkephalin	Tyr-Gly-Gly-Phe-Met
[D-Ala^2, D-Leu^5] Enkephalin	Tyr-D-Ala-Gly-Phe-D-Leu

Peptide Name	Sequence
[Leu^5] Enkephalin	Tyr-Gly-Gly-Phe-Leu
[D-Ala^2, D-Met^5] Enkephalin	Tyr-D-Ala-Gly-Phe-D-Met

Heterocyclic aromatic amines (HAAs) constitute a complex group of compounds belonging to two main chemical classes: aminocarbolines and aminoimidazaarenes. They are formed in trace quantities from protein-rich foods when processed by thermal treatments such as typical cooking practices (31). HAAs are included among the large number of chemicals that have been found to be potential carcinogens. They are found to be carcinogenic in rodents and are candidates to cause common human cancer as well (32). The structures of all the HAAs used in this study are shown in the Figure 8 along with their chemical names. These compounds were tested only on some of the capillaries etched with salts and capillaries etched with salts and then chemically modified.

3.5 Comparison of Bare Capillary, Capillaries Etched With Salts and Capillaries Etched With Salts and Chemically Modified

All four types of capillaries i.e., bare, etched with salts, etched with salts and chemically modified and etched and chemically modified without salts gave better separation of the mixture of enkephalins at the acidic pH values (2.14, 3.00, 3.7 and 4.41). In some instances the separation is improved by the addition of an organic modifier like methanol or acetonitrile in the buffer solvent. Most of the capillaries exhibited better resolution at pH

3.00 and 3.7 though the solute species are resolved at pH values of 2.14 and 4.41. The order of migration is Met^5 enkephalin, D-Ala^2, D-Met^5 enkephalin and Leu^5 enkephalin. A change in the order of migration is seen in the capillary etched with calcium nitrate. Migration times of the capillaries etched with the salts are shorter than their respective capillaries etched with salts and chemically modified though the lengths of the capillaries are almost the same. Better peak shapes are exhibited by the capillaries that have been etched with salts and chemically modified than the capillaries etched only with salts. Addition of organic modifiers resulted in better resolution and peak symmetries where satisfactory results were not achieved by buffers alone.

The migration times on the bare capillary are very long. Though the solute species are well resolved the peak symmetry is very poor (Figure 20A). Separation is achieved at low pH values like 2.14, 3.00, 3.70. At buffer pH values above 3.7 the peaks co-migrate (Figure 20B). Peak symmetry has improved considerably but the resolution is lost. This is the normal behavior expected for protein and peptides on the bare capillary. A bare capillary has many free silanol groups that deprotonate. Enkephalins which are short peptides adsorb onto these deprotonated silanol groups. At low pH the amine groups of the enkephalins are positively charged which adhere to the negatively charged surface of the capillary taking a longer time to migrate and poor peak shapes are observed.

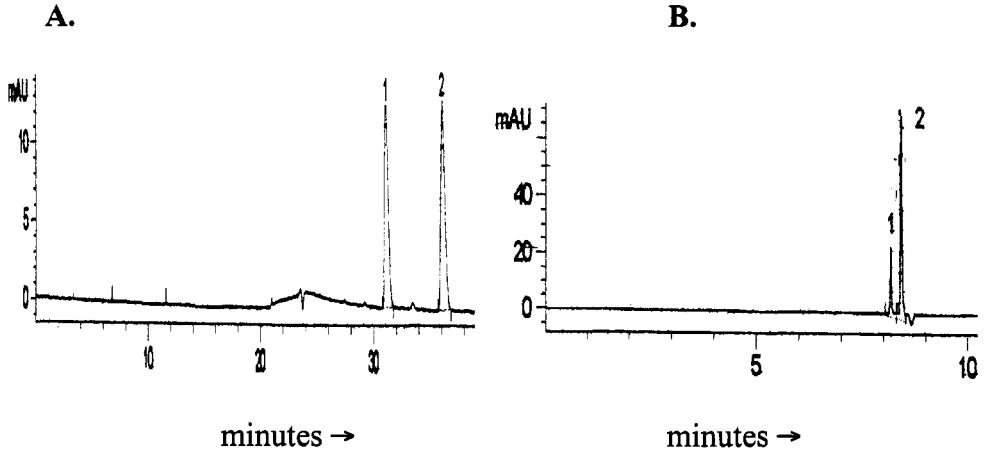


Figure 20 – Separation of Two Enkephalins on Bare Capillary (pH 2.14 and 4.41).

Separation of Met^5 Enkephalin and Leu^5 Enkephalin.
Bare Capillary. L = 64.5 cm, l = 56.5 cm, V = 25 kV, at 210 nm.
A - pH 2.14 (1:10), B - pH 4.41 (1:10)
1. Met^5 Enkephalin
2. Leu^5 Enkephalin

Moreover the low EOF developed at lower pH gives ample on-capillary time for the enkephalins to adsorb onto the surface leading to the greater peak widths. As the pH increases more silanols lose protons resulting in higher EOF. This leads to a decrease in on-capillary time of the solute species thereby resulting in less adsorption of the solute species to the capillary inner surface. Hence the peak shape has improved but the resolution is lost. At pH values of 7.06 and 8.14 the solute species are deprotonated bearing only negative charge on them due to the carboxylate of the peptide. Under these conditions they don't adsorb onto the surface that is also negatively charged but they co-migrate.

The sample mixture is well resolved with good peak shape with capillaries etched in the absence of salts and later chemically modified. The chemical modification by bonding of an octadecyl group to the Si-H intermediate results in the presence of fewer free silanol groups on the surface. The adsorption of the solute species onto the surface is reduced and hence better peak shapes are obtained. Moreover, the low EOF developed due to fewer silanols gives the solute more on-capillary time for better interaction with the stationary phase for better resolution.

The capillaries etched with inorganic salts and those which are also subsequently chemically modified have exhibited a different pattern from that described above. The presence of each salt has given unique characteristics to their respective etched capillary and etched chemically modified capillary. The etched and etched chemically modified capillaries with cupric chloride salt gave good resolution of the solute species. In the case of the etched capillary with $CuCl_2$ better peak symmetry is achieved (Figure 21). The peak symmetry on the etched chemically modified capillary with $CuCl_2$ is very poor with large peak widths (Figure 22).

If the symmetry of the peaks is good on the etched chemically modified capillary in the absence of salts then the presence of the $CuCl_2$ has something to do with the separation process. The large peak tailing with greater peak widths indicate the strong adsorption of the solute species onto the surface of the capillary.

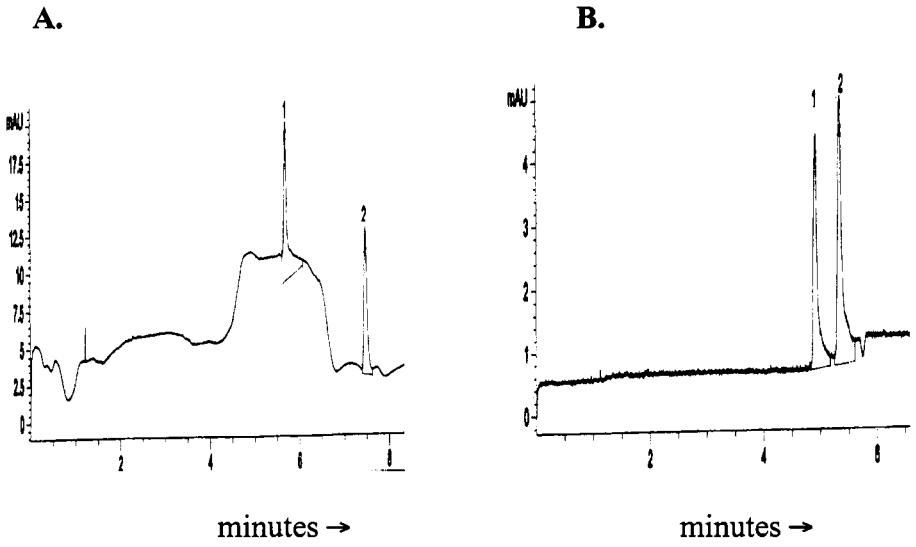


Figure 21 – Separation of Two Enkephalins on Capillary Etched with $CuCl_2$ (pH 4.41 and pH 3.7).

Separation of Met^5 Enkephalin and Leu^5 Enkephalin on etched capillary with $CuCl_2$. L = 36.5 cm, l = 28 cm, V = 25 kV, 210 nm. A - pH 4.41 (1:10), B - pH 3.7 (1:10).
1. Met^5 Enkephalin; 2. Leu^5 Enkephalin

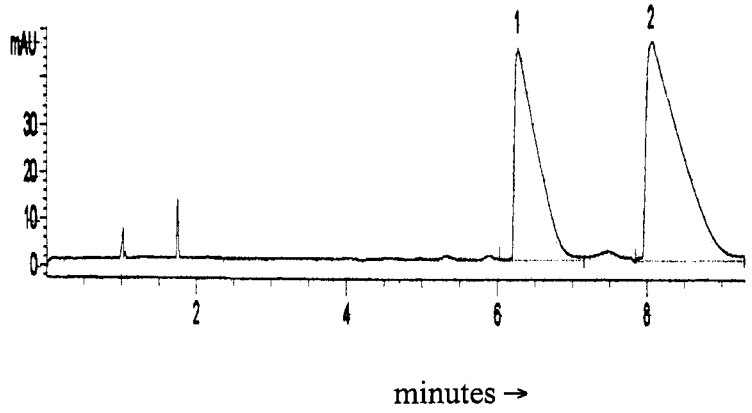


Figure 22 – Separation of Two Enkephalins on Capillary Etched with $CuCl_2$ and C18 Modified (pH 2.14).

Separation of Met^5 Enkephalin and Leu^5 Enkephalin. Etched and chemically modified capillary with $CuCl_2$ salt. L = 33 cm, l = 24 cm, V = 25 kV, 210 nm, pH = 2.14 (1:10).
1. Met^5 Enkephalin; 2. Leu^5 Enkephalin

It could be first due to the stronger solute stationary phase interactions or secondly, due to the interaction with the inorganic ions in the surface matrix of the capillary. If the first reason is true then the peak tailing should be seen in the etched chemically modified capillary without additional salts, but that is not observed. If the second reason is true then the same pattern should be seen in the etched capillary with $CuCl_2$ salt to some extent. This is still unexplainable at this point. It could be a combined effect also. The presence of the cupric chloride ions might have obstructed efficient bonding of C18 groups to the hydrides. The result is the presence of Si-H groups on the surface without being modified, which later convert to free silanol groups when they come in contact with water. These Si-OH groups on the capillary surface could be responsible for the strong adsorption of the solute species leading to a poor peak shape.

The etched capillary with ammonium carbonate salt gave good separation for the mixture of enkephalins at low pH up to 3.70. The best resolution is achieved at pH 3.00 (Figure 23A). At pH 2.14 there is no baseline separation of the first two peaks. It may be that the electrophoretic mobilities of the two species could be similar at that pH. Similarly at pH 4.41 baseline separation is not achieved (Figure 23B). The etched chemically modified with the same salt shows good resolution at pH 3.00, 3.7 and 4.41 (Figure 24). The peaks are well resolved with better peak shapes than the etched capillary with the same salt. In fact the best separation is achieved at

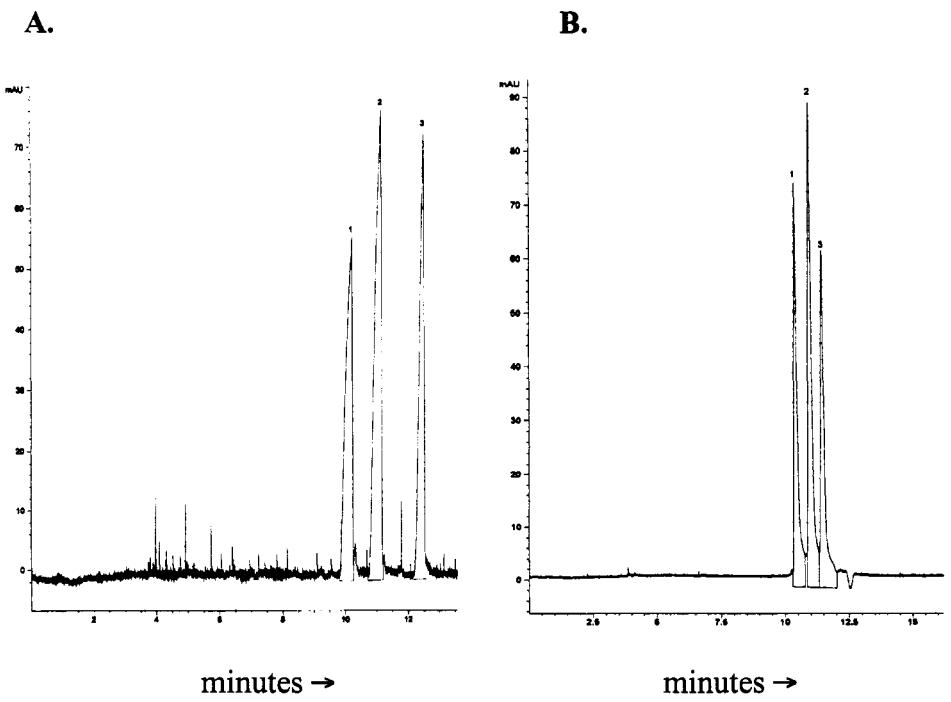


Figure 23 – Separation of Three Enkephalins on Capillary Etched with $(NH_4)_2CO_3$ (pH 3.00, pH 4.41).

Separation of Met^5 Enkephalin, D-Ala^2, D-Met^5 Enkephalin and Leu^5 Enkephalin. L = 58 cm, l = 49.5 cm, V = 25 kV, 210 nm.
A - pH 3.00 (1:10), B – pH 4.41 (1:10).
1. Met^5 Enkephalin; 2. D-Ala^2, D-Met^5 Enkephalin; 3. Leu^5 Enkephalin.

pH 3.00 in both the etched and etched chemically modified capillary with ammonium carbonate because a small impurity peak at the base of the first peak is well resolved when compared to the results at pH 3.7 and 4.41. Though there is resolution at pH 3.7 the peaks have some tailing. The difference in the peak shape at these two pH values cannot be due to the presence of inorganic salt alone. If that was the reason then the tailing should be seen at pH 3.00 too. Also it cannot be purely due to the number of silanols

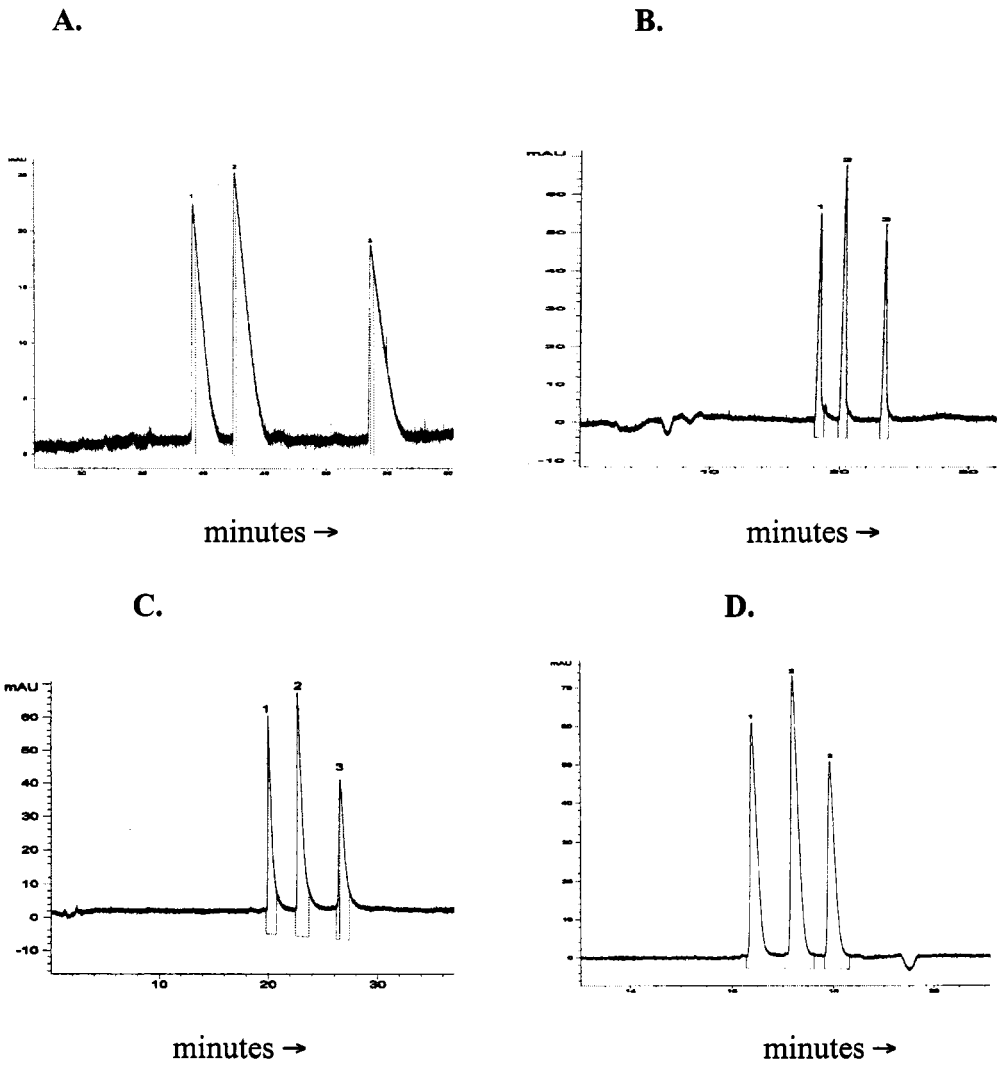


Figure 24 – Separation of Three Enkephalins on Capillary Etched with $(NH_4)_2CO_3$ and C18 Modified. (pH 2.14, 3.00, 3.7, 4.41).

Separation of Met^5 Enkephalin, D-Ala^2, D-Met^5 Enkephalin and Leu^5 Enkephalin. salt. L = 57 cm, l = 48.5 cm, V = 25 kV, 210 nm. A - pH 2.14 (1:10), B – pH 3.00 (1:10), C - pH 3.70 (1:10), D – pH 4.41 (1:10).
1. Met^5 Enkephalin; 2. D-Ala^2, D-Met^5 Enkephalin; 3. Leu^5 Enkephalin

present on the surface as the difference may not be significant enough to change the peak shape. But it could be due to minor differences in the

ionization of the solute species at two different pH values. Moreover, the citrate buffer (pH 3.00) has more conductivity than the lactate buffer (pH 3.7). Higher conductivity results in higher EOF hence a decrease in on-capillary time of the solute species for adsorption to the surface.

The resolution improved at pH 2.14 and 4.41 from the capillary etched only with ammonium carbonate to the capillary etched with ammonium carbonate and chemically modified. The EOF is lower in the capillary etched with ammonium carbonate and chemically modified than in the capillary etched only with the same salt. This allows for more solute stationary interactions and better resolution. But the resolution as well as peak shape improved at pH 4.41 in the capillary etched with ammonium carbonate and chemically modified.

The other noticeable point is that the migration times have increased in the capillary etched and chemically modified than in the capillary etched only with ammonium carbonate, which is in agreement with the calculations that were discussed in the EOF section. This indicates that only a few Si-OH groups remained on the surface after bonding the organic moiety.

Furthermore the capillary etched with ammonium carbonate and chemically modified showed better efficiency than the capillary etched with cupric chloride and chemically modified. The presence of the ammonium carbonate might have improved the bonding of the C18 group, which better

shielded the remaining silanol groups on the surface. The presence of more alkyl groups led to a less adsorption of the solutes onto the surface.

The capillary etched only with calcium nitrate and the capillary etched with calcium nitrate and chemically modified capillary demonstrated a unique behavior of their own. Resolution is not achieved even at low pH. There is no baseline separation at any pH from 2.14 to 4.41. In addition there is a change in the selectivity from pH 2.14 to pH 3.7 to pH 4.41 in the case of the capillary etched with $Ca(NO_3)_2$ (Figure 25). Better peak shape and resolution is accomplished in the capillary etched with $Ca(NO_3)_2$ and chemically modified at pH 3.00 with the addition of an organic modifier to the mobile phase (Figure 26).

Usually the addition of an organic modifier affects the overall pH of the buffer which might have affected the ionization of the solute species resulting in a change in the selectivity of the solute species. Furthermore some extra impurity peaks or some unknown peaks are also present along with the solute peaks. One likely explanation is that the inorganic ions must be present near the surface matrix closer to the analytes leading to better interaction of the solute species with these ions.

In addition the low EOF in the capillary etched with $Ca(NO_3)_2$ results in longer on-capillary time for the solutes, enhancing the interaction with the surface matrix. But the capillary etched with the same salt, $Ca(NO_3)_2$ and

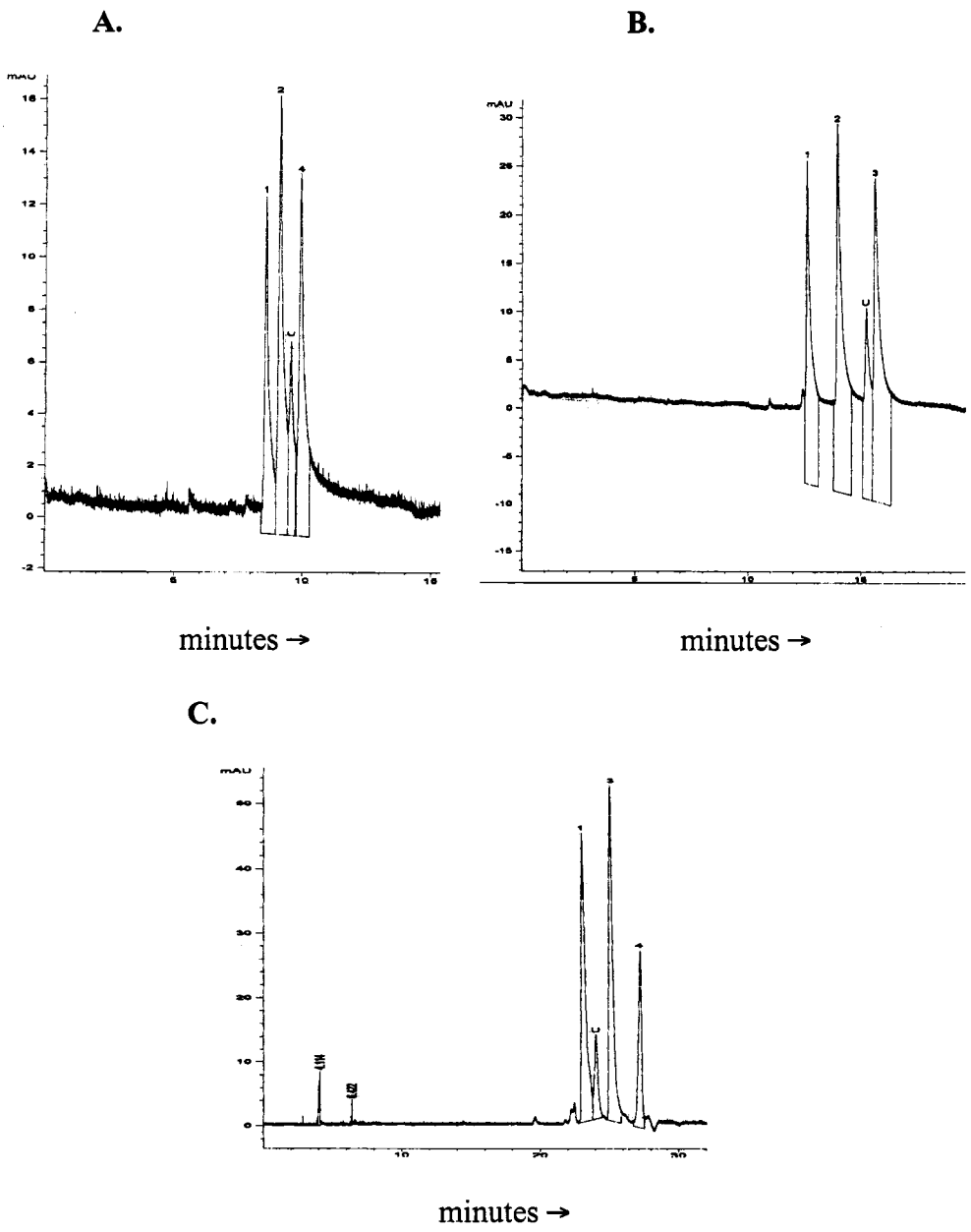


Figure 25 – Separation of Three Enkephalins on Capillary Etched with $Ca(NO_3)_2$ (pH 3.00, 3.7 and 4.41).

Separation of Met[5] Enkephalin, D-Ala[2], D-Met[5] Enkephalin and Leu[5] Enkephalin. L = 66 cm, l = 57.5 cm, V = 25 kV, 210 nm.
A - pH 3.00 (1:10), B – pH 3.70 (1:10), C - pH 4.41 (1:10).
1. Met[5] Enkephalin; 2. D-Ala[2], D- Met[5] Enkephalin;
3. Leu[5] Enkephalin; U. Unknown

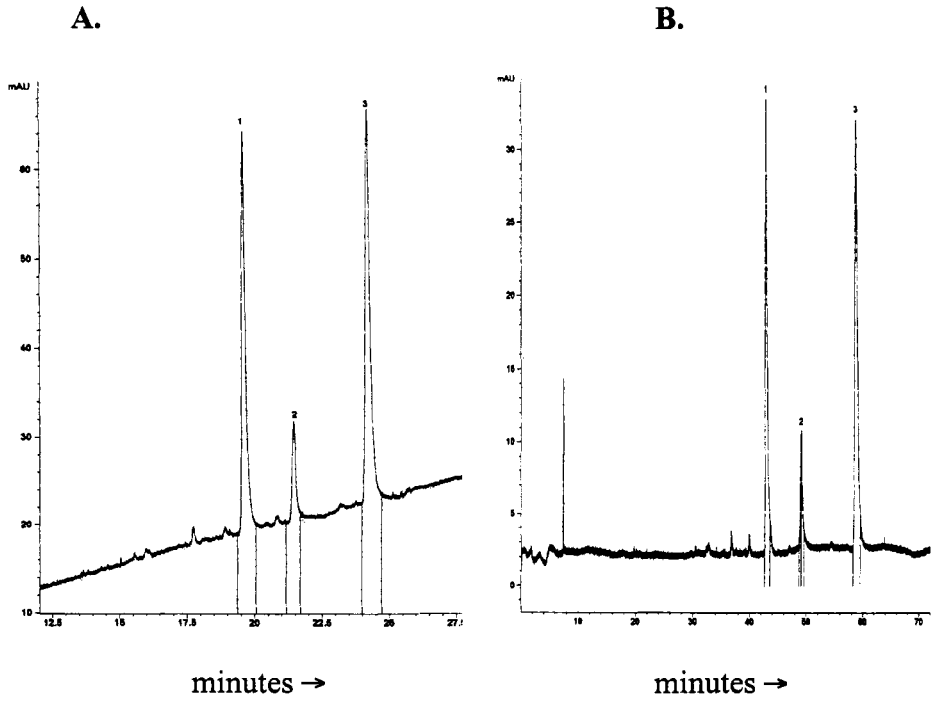


Figure 26 – Separation of Three Enkephalins on Capillary Etched with $Ca(NO_3)_2$ and C18 Modified. (pH 3.7 + 50% Organic Modifier)

Separation of Met^5 Enkephalin, $D\text{-}Ala^2$, $D\text{-}Met^5$ Enkephalin and Leu^5 Enkephalin. L = 64 cm, l = 57.2 cm, V = 25 kV, 210 nm.
A - pH 3.70 (1:10) + 50% MeOH, B – pH 3.70 (1:10) + 50% ACN.
1. Met^5 Enkephalin; 2. $D\text{-}Ala^2$, $D\text{-}Met^5$ Enkephalin; 3. Leu^5 Enkephalin

chemically modified showed better resolution and improved peak symmetry than its respective etched capillary. The reason could be that after modification there are long alkyl groups radially extending into the center of the capillary. These C18 groups are in close proximity to the solute species avoiding the interactions of solute species with the inorganic ions in the surface matrix. Hence, those unknown peaks that were observed in the etched only capillary are not seen in the case of the etched chemically modified

capillary with calcium nitrate salt. This once again proves that the existence of the additional salts in the surface matrix has definitely some effect on the separation process.

The capillary etched with $CrCl_3$ and the capillary etched with the same salt and chemically modified displayed some differences from the capillary etched with no additional salts and chemically modified. Separation is again attained only at low pH between 2.14 to 4.41 though no baseline separation is seen at pH 4.41 (Figure 27). The etched capillary gave good resolution but poor peak shape. Either fronting or tailing is observed. The peak widths are significantly greater. This indicates that there is some interaction of the solute species with the $CrCl_3$ ions present in the matrix. The interactions could be ionic or hydrophilic. In addition there could be some mass transfer effects.

The peak shapes have improved in the capillary etched with $CrCl_3$ and chemically modified but the best symmetry is attained by the addition of organic modifier to the buffer solvent. The organic modifier changes the zeta potential thereby reducing the EOF and improving resolution.

Capillaries etched with $NaNO_3$ and etched as well as chemically modified gave good resolution and peak symmetries at low pH like all the other capillaries (Figure 28). The peak symmetry is excellent in both the etched and etched chemically modified capillaries but comparatively better at pH 3.7 than at pH 3.00. A four-sample mixture is separated on the above mentioned capillaries.

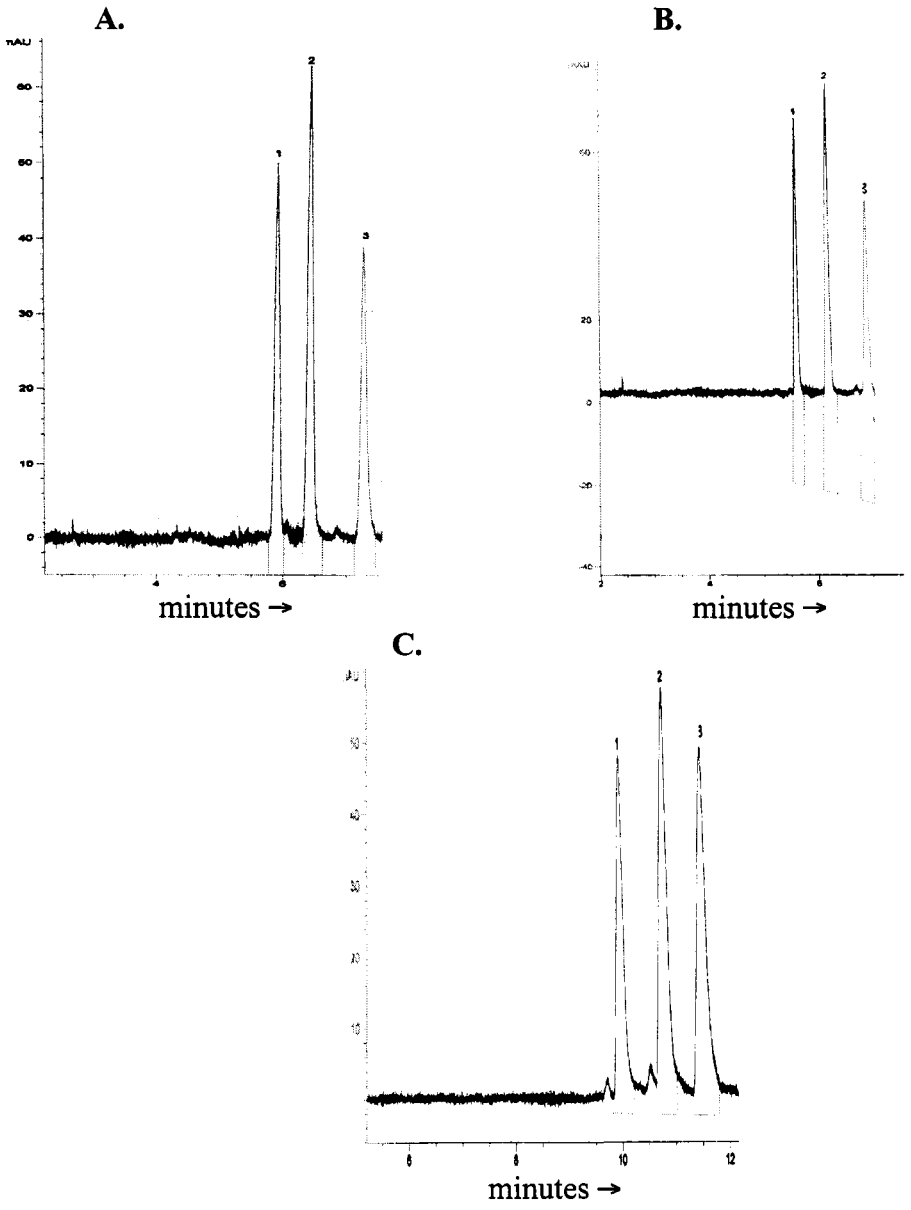


Figure 27 – Separation of Three Enkephalins on Capillary Etched with $CrCl_3$ and C18 Modified (pH 3.00, 3.7 and 4.41)

Separation of Met^5 Enkephalin, D-Ala^2, D-Met^5 Enkephalin and Leu^5 Enkephalin. L = 47 cm, l = 38.5 cm, V = 25 kV, 210 nm.
A – pH 3.00 (1:10), B – pH 3.7 (1:10), C – pH 4.41 (1:10)
1. Met^5 Enkephalin; 2. D-Ala^2, D-Met^5 Enkephalin; 3. Leu^5 Enkephalin

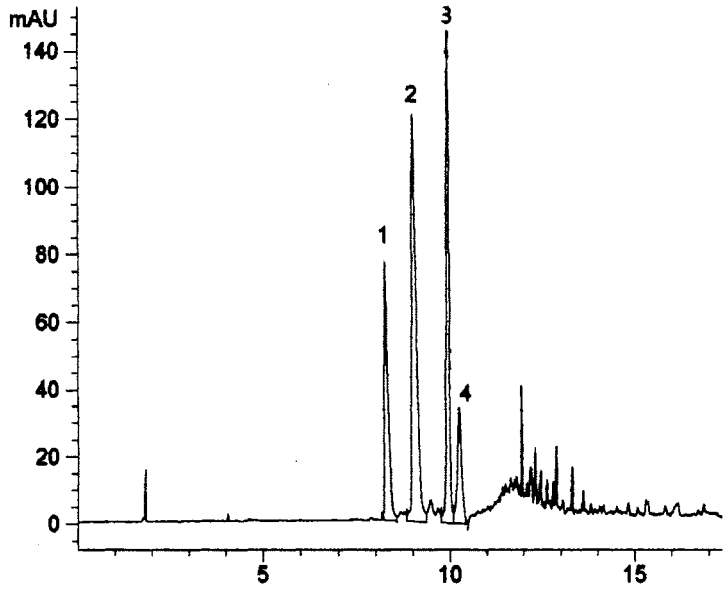


Figure 28 – Separation of Four Enkephalins on Capillary Etched with $NaNO_3$ (pH 4.41).

Separation of Met^5 Enkephalin, D-Ala^2, D-Met^5 Enkephalin, D-Ala^2, D-Leu^5 Enkephalin and Leu^5 Enkephalin. L = 67.5 cm, l = 59 cm, V = 25 kV, pH = 4.41 (1:10), 210 nm.
1. Met^5 Enkephalin; 2. D-Ala^2, D-Met^5 Enkephalin;
3. D-Ala^2, D-Leu^5 Enkephalin; 4. Leu^5 Enkephalin

Some unknown peaks are seen could be yet again because of the interaction of the solute species with the existent inorganic ions in the surface matrix (Figure 29). May be the interaction is not that strong enough as is seen in capillaries etched with other salts so there are enhanced peak shapes and resolution. Or the presence of fewer ions could also be a reason as the salt is sparingly soluble in the etching solution. Consequently fewer interactions and improved peak shapes are obtained.

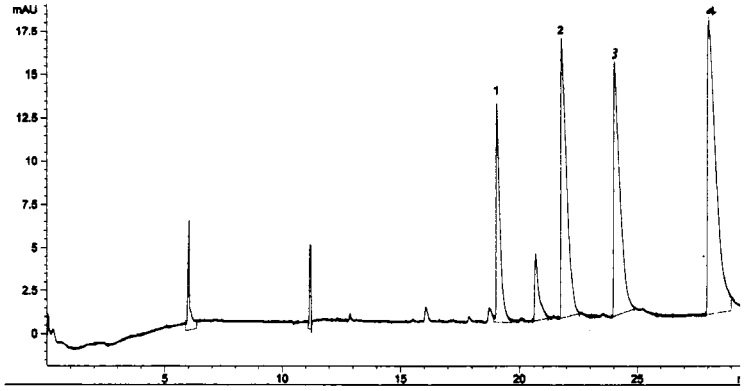


Figure 29 – Separation of Four Enkephalins on Capillary Etched with $NaNO_3$ (pH 3.00 + 50% ACN).

Separation of Met^5 Enkephalin, D-Ala^2, D-Met^5 Enkephalin, D-Ala^2, D-Leu^5 Enkephahlin and Leu^5 Enkephalin. L = 67.5 cm, l = 59 cm, V = 25 kV, 210 nm. pH = 3.00 (1:10) + 50% ACN. 1. Met^5 Enkephalin; 2. D-Ala^2, D-Met^5 Enkephalin; 3. D-Ala^2, D-Leu^5 Enkephalin; 4. Leu^5 Enkephalin.

Finally, the additional capillary etched with $CuCl_2$ and chemically modified not treated with 0.1 M NaOH solution gave a completely different response when compared to the etched chemically modified capillary with $CuCl_2$ treated with 0.1 M NaOH. Though the migration times are comparable better symmetry is obtained with the former capillary. Baseline resolution and good peak shapes were found at pH 3.00 and 3.7 (Figure 30). If the poor peak shape in the capillary etched with $CuCl_2$ and chemically modified is a result of the adsorption to the Si-OH groups due to the poor bonding of the C18 leaving many hydrides which later convert to Si-OH, then skipping the

treatment with 0.1 M NaOH solution might have helped in better resolution and efficiency.

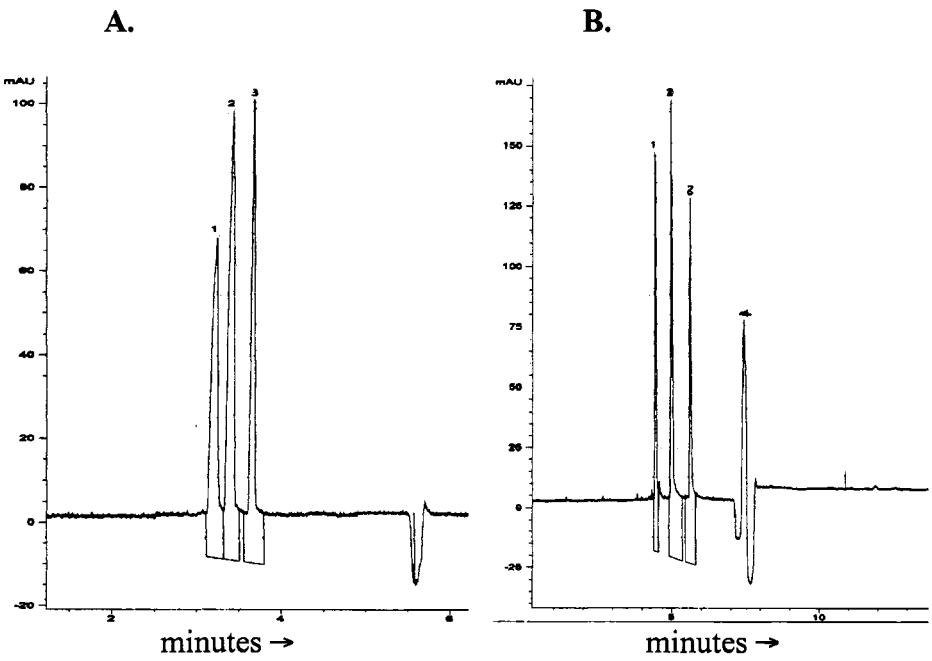


Figure 30 – Separation of Three Enkephalins on Capillary Etched with $CuCl_2$ and C18 Modified without 0.1 M NaOH (pH 3.00 and 3.7).

Separation of Met^5 Enkephalin, D-Ala^2, D-Met^5 Enkephalin, and Leu^5 Enkephalin. L = 36 cm, l = 27.5 cm, V = 25 kV, 210 nm. A - pH 3.00 (1:10), B - pH 3.7 (1:10) + 50% ACN 1. Met^5 Enkephalin; 2. D-Ala^2, D-Met^5 Enkephalin; 3. Leu^5 Enkephalin

In other words leaving out the 0.1 M NaOH step might not have restored the Si-OH groups that are usually lost in the etching process. So even after bonding many silanols are not present reducing the adsorption and enhancing the peak symmetry when compared to the capillary that was treated with 0.1M NaOH though the same salt is present.

HAAs were separated only on some capillaries as a means of assessing different solutes. Five compounds were tested on the bare capillary, a capillary etched with $CuCl_2$ and a capillary etched with the same inorganic salt and chemically modified. The best separation was obtained at low pH. The order of migration is MeIQ, IQ, AαC, MeIQx and PhIP. On the bare capillary complete separation is achieved at pH 2.14 and 3.00. Above pH 3.00 there is overlapping of the peaks. The first two peaks co-migrated at all pH values but some separation is seen at pH 2.14 (Figure 31A). Above pH 2.14 separation of the first two peaks is not seen. At pH 3 And 3.7, the second and third peaks co-migrated into a single peak (Figure 31B).

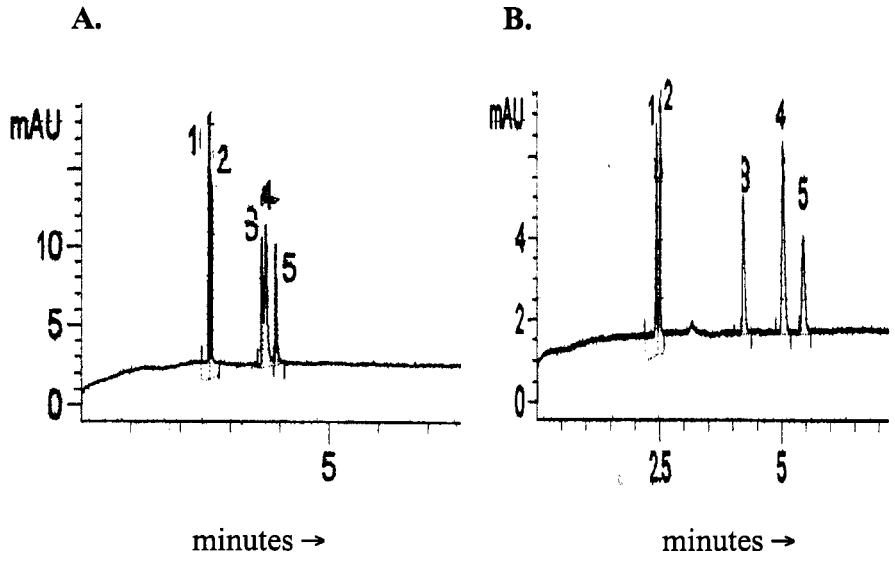


Figure 31 – Separation of Five HAA's on Bare Capillary (pH 3.7 and 2.14).
Separation of 1. MeIQ, 2. IQ, 3. AαC, 4. MeIQx and 5. PhIP. L = 64.5 cm, l = 56.5 cm, V = 25 kV, 263 nm. A - pH 3.70 (1:10), B – pH 2.14 (1:10).

Above pH 4.41 the order of the migration has changed: MeIQ and IQ as a single peak; MeIQx; PhIP; and AαC. On the capillary etched with $CuCl_2$ again separation is found at low pH, 2.14 and 3.00 (Figure 32).

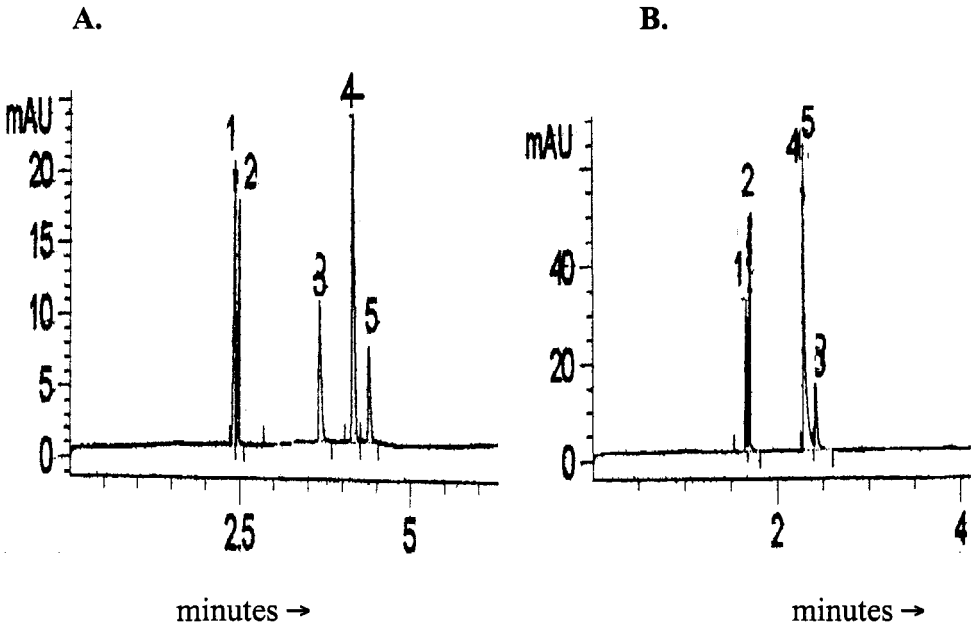


Figure 32 – Separation of Five HAA's on Capillary Etched with $CuCl_2$ (pH 2.14 and pH 3.7)

Separation of MeIQ, IQ, AαC, MeIQx and PhIP.
L = 36.5 cm, l = 28 cm, V = 25 kV, 263 nm.
A - pH 2.14 (1:10), B – pH 3.70 (1:10).
1. MeIQ; 2. IQ; 3. AαC ; 4. MeIQx; 5.PhIP

According to the previous work of Pesek and coworkers better peak shape was achieved on the etched capillary (20). But here though there is resolution of the peaks the peak shapes are not satisfactory. So it points out that the presence of the salt has some effect on the separation of the solute species. The capillary etched with $CuCl_2$ and chemically modified did not

produce good peak shapes. The resolution is not that satisfactory (Figure 33). This could be due to the adsorption of amines onto the bonded moiety or onto the surface.

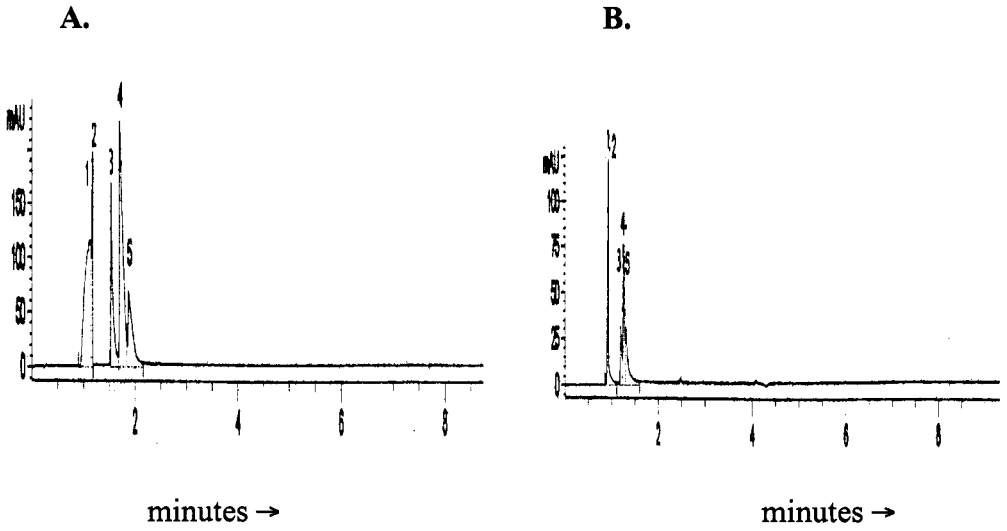


Figure 33 – Separation of Five HAA's on Capillary Etched with $CuCl_2$ and C18 Modified (pH 2.14 and 3.00).

Separation of MeIQ, IQ, Aœc, MeIQx and PhIP.
L = 33 cm, l = 24.5 cm, V = 25 kV, 263 nm.
A - pH 2.14 (1:10), B - pH 3.00 (1:10)
1. MeIQ; 2. IQ; 3. AœC; 4. MeIQx; 5. PhIP

3.6 Effect of Organic Solvent on the Separation of Compounds

Addition of organic modifiers like methanol and acetonitrile change the magnitude of the EOF. The potential difference developed between the two layers formed at the surface by a buffer under the influence of an applied voltage is called the zeta potential. When the organic modifiers are added the non-polar solvents change the ionization of the buffers thus changing the

formation of the double layer. This alters the zeta potential leading to a diminished EOF. The presence of organic modifiers also reduces the viscosity of the solvent thereby increasing the electrophoretic mobility of the species. The migration times were very long in such circumstances where organic modifier, either methanol or acetonitrile was present. This is true for all capillaries irrespective of the type of salt present and whether the capillary is only etched or etched and chemically modified. In most of the cases the migration times with 50% methanol are much longer when compared to that of 50% acetonitrile (Figure 34). Because the EOF is small in the presence of

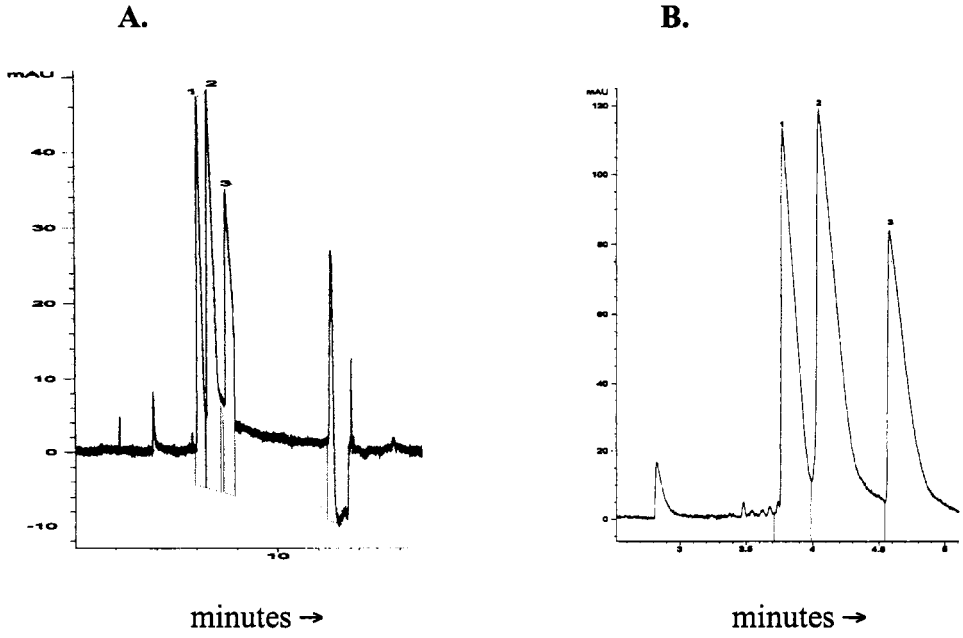


Figure 34 – Separation of Three Enkephalins on Capillary Etched with $CrCl_3$ (pH 3.00 + 50% MeOH and 3.00 + 50% ACN).

Separation of Met^5 Enkephalin, D-Ala^2, D-Met^5 Enkephalin and Leu^5 Enkephalin. L = 36 cm, l = 28 cm, V = 25 kV, 210 nm.
A – pH 3.00 (1:10) + 50% MeOH, B – pH 3.00 (1:10) + 50% ACN
1. Met^5 Enkephalin; 2. D-Ala^2, D-Met^5 Enkephalin;
3. Leu^5 Enkephalin

methanol in the mobile phase the solute species are well resolved in some instances. Whereas in the presence of acetonitrile in the mobile phase the EOF is higher and there is a loss of resolution. The reason for higher EOF with acetonitrile in the etched capillaries may be due to the fact that the inner surface of the capillary becomes more polar in the presence of the ions in the surface matrix. So acetonitrile being more non-polar than methanol, the ions will migrate faster. In the same manner methanol will tend to remain for longer time in the capillary due to the solvent wall interactions. Thus the migration time can be affected depending on the solvent leading to better resolution in the case of methanol and loss of resolution in the case of acetonitrile.

Capillaries etched with salts and chemically modified should show a different behavior. They now have an organic moiety hence should exhibit a more non-polar nature. Then the addition of acetonitrile should result in lower EOF because solvent wall interactions are non-polar. Instead, they have displayed the same behavior as etched capillaries with the salts. So this once again indicates that the surface of the capillary is polar rather than being non-polar. This unusual nature of the capillaries etched with salts and subsequently chemically modified may possibly be caused by the presence of the salts. Maybe the ions of the inorganic salts must be present superficially making the surface of the capillary polar or affecting the bonding by C18 group.

However there are exceptions to this situation. In the capillaries etched with $CrCl_3$ and $Ca(NO_3)_2$ and those etched with the same salts and chemically modified, the migration times are shorter with the addition of methanol than with the addition of acetonitrile (Figure 35). In this case

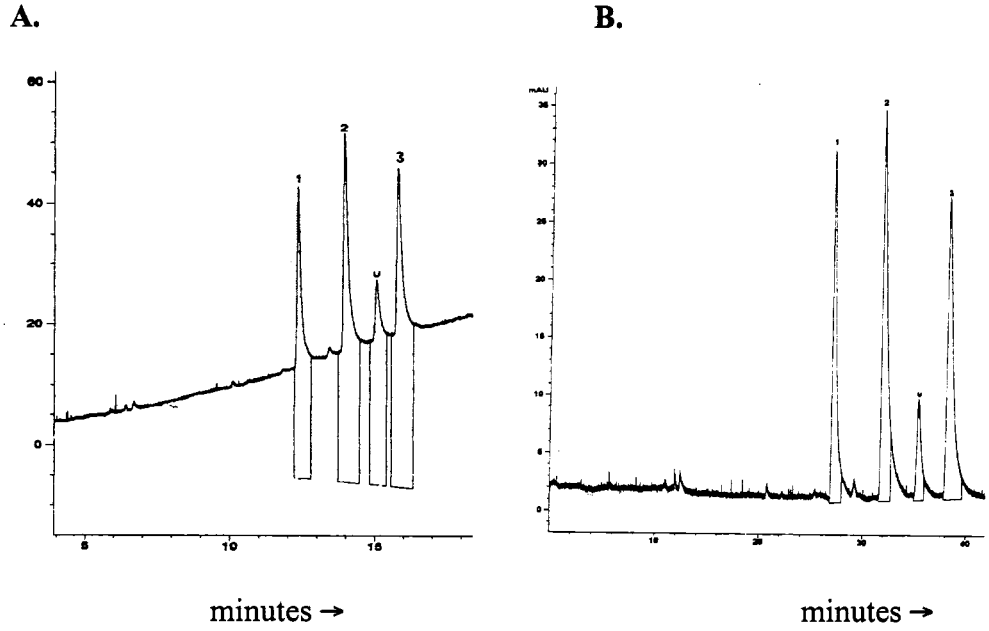


Figure 35 – Separation of Three Enkephalins on Capillary Etched with $Ca(NO_3)_2$ (pH 3.7 + 50% MeOH and 3.7 +50% ACN).

Separation of Met^5 Enkephalin, D-Ala^2, D-Met^5 Enkephalin and Leu^5 Enkephalin. L = 66 cm, l = 57.5 cm, V = 25 kV, 210 nm.
A – pH 3.7 (1:10) + 50% MeOH, B – pH 3.7 (1:10) + 50% ACN
1. Met^5 Enkephalin; 2. D-Ala^2, D-Met^5 Enkephalin; 3. Leu^{5}Enkephalin

the ions may be present deeper in the surface matrix or there may be different types of surface interactions with methanol than with the acetonitrile. Methanol and acetonitrile did show some changes in the resolution of the

compounds. Usually, the buffers with no organic modifier gave good separations. But in some case where the compounds co-migrated, organic modifiers did improve resolution.

Capillaries that were etched with $Ca(NO_3)_2$ and etched with the same salt and chemically modified did not give good separation of the sample mixture with buffer alone. In this case the presence of an organic modifier gave well-separated peaks when compared to the analyses with no organic modifier (Figure 36).

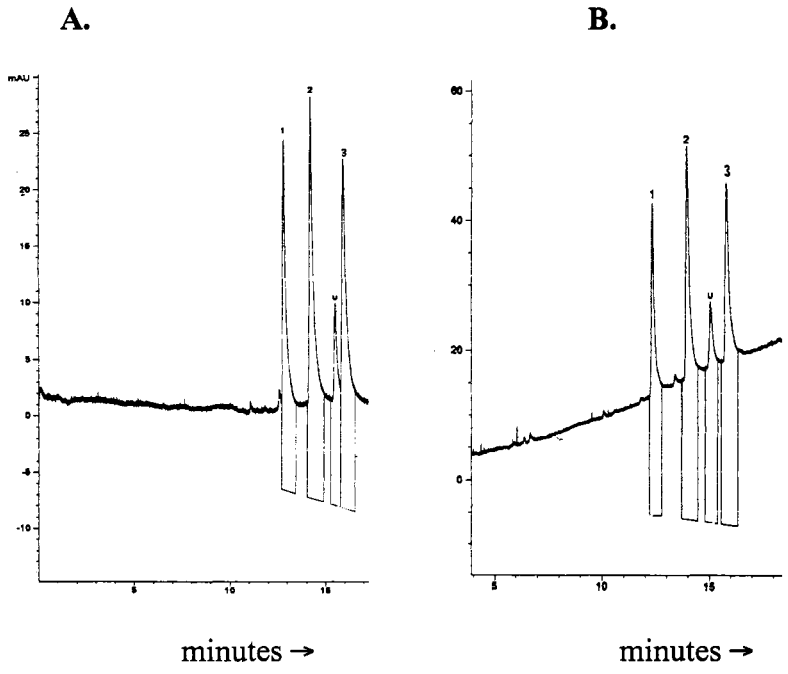


Figure 36 – Separation of Three Enkephalins on Capillary Etched with $Ca(NO_3)_2$ (pH 3.7 and 3.7 + 50% ACN).

Separation of Met^5 Enkephalin, D-Ala^2, D-Met^5 Enkephalin and Leu^5 Enkephalin. L = 66 cm, l = 57.5 cm, V = 25 kV, 210 nm. A – pH 3.7 (1:10), B – pH 3.7 (1:10) + 50% MeOH
1. Met^5 Enkephalin; 2. D-Ala^2, D-Met^5 Enkephalin;
3. Leu^5 Enkephalin

The organic modifier usually changes the surface ionization by changing the zeta potential and lowers the EOF. This affects not only the solute wall / stationary phase interactions but also gives more on-capillary time for the solutes leading to better interactions with the stationary phase.

The peak shape improved in the capillary etched with ammonium carbonate with the addition of methanol or acetonitrile in the buffer (Figure 37). Again the addition of the organic modifier lowers the EOF resulting in solute stationary phase interactions leading to better resolution.

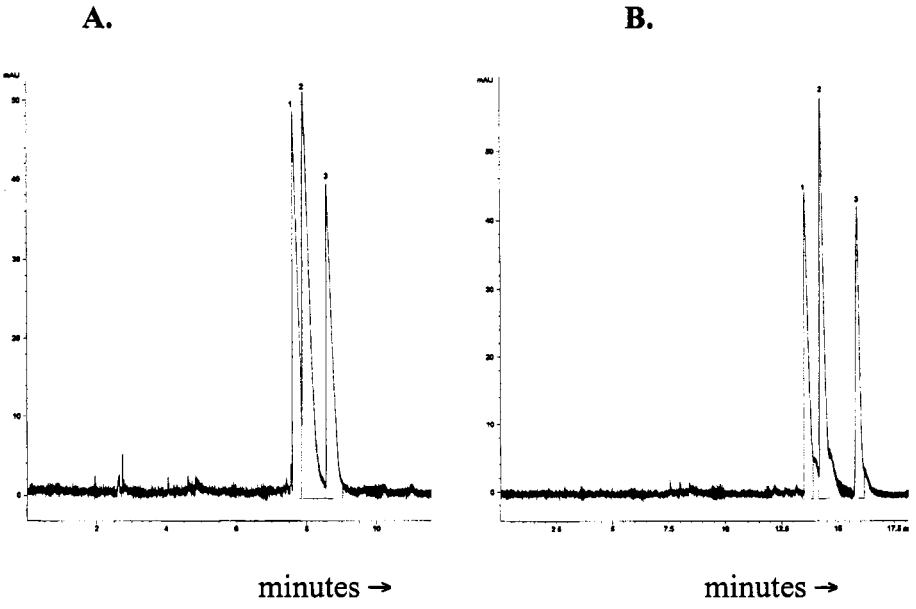


Figure 37 – Separation of Three Enkephalins on Capillary Etched with $(NH_4)_2CO_3$ (pH 2.14 and 2.14 + 50% MeOH).

Separation of Met^5 Enkephalin, D-Ala^2, D-Met^5 Enkephalin and Leu^5 Enkephalin. L = 58 cm, l = 49.5 cm, V = 25 kV, 210 nm.
A - pH 2.14 (1:10), B - pH 2.14 (1:10) + 50% MeOH
1. Met^5 Enkephalin; 2. D-Ala^2, D-Met^5 Enkephalin
3. Leu^5 Enkephalin

In most of the instances the migration times are longer in the presence of organic modifiers in the mobile phase when compared to the buffers with no organic modifiers. In certain circumstances the sample peaks have migrated such that they are better resolved when compared to their respective buffers in the absence of organic modifiers. In general, the employment of organic modifiers, either methanol or acetonitrile, have affected the resolution and symmetry of the peaks during the course of separation of the sample mixture. The effects could be due to the change in zeta potential thereby lowering the EOF. The lower EOF allows solute / stationary phase interactions giving way to improved resolution. Perhaps it is also possible that the organic modifier might be interacting with the inorganic ions in the surface matrix apart from changing the zeta potential resulting in better resolution, or some combination of both effects.

Peptides are often difficult to separate on a bare capillary. Successful separation is achieved by the etching and modification of the capillaries. The etched chemically modified capillary without salts did show improvement in comparison to the bare capillary in the separation of the sample mixture. The migration times are shorter; the solute species are well resolved with good peak symmetry. But the investigation of the presence of inorganic salts in the surface matrix of the glass did indicate a difference. The presence of cupric chloride changed the peak shape. The peaks displayed considerable tailing. Capillaries etched with $CrCl_3$ and $(NH_4)_2CO_3$ and chemically modified gave

good separation similar to the etched chemically modified without salts. In a comparison between chloride salts and nitrate salts, the capillaries etched with nitrate salts like $NaNO_3$ and $Ca(NO_3)_2$ and chemically modified exhibited longer migration times. It may be that the chloride salts led to a stronger double layer creating a larger EOF. During the course of preparation of the saturated solution of the salt with the etching agent it was observed that the nitrate salts were less soluble than the chloride salts. If there were fewer ions in the surface matrix as a result of lower solubility then the capillary should act like the normal C-18 capillary. But that is not observed here. This result indicates that some change in the surface morphology of the capillary is causing the sample compounds to interact differently with it. Moreover in the case of nitrate salts an extra-unknown peak is seen. Perhaps the ions in the surface of the capillary are interacting with the sample and help to resolve the impurities in the sample. Overall there is a considerable affect of the inorganic ions on the surface matrix of the capillary, in the creation of EOF and in the process of sample separation. In some instances it led to a better resolution, in others it didn't. In some it affected the selectivity as well.

The change in the migration times and selectivity of the compounds could be due to the solute behavior also. The sample species migrate according to their ionization based on the pH of the buffer used apart from moving along with EOF. The change in the pH of the buffer or the

addition of the organic modifier may cause alteration in the net charge of the molecule. This affects the selectivity and migration times of the compounds.

Table 4: Results and Observations of Separation of Enkephalins on all Types of Capillaries.

Capillary Type Or Salt Used	Results and Observations	
Bare Capillary	Resolution at low pH; Co-migrated above pH 3.00; Long migration times poor peak symmetry	
	Etched	Etched and C18 Modified
Cupric Chloride	Resolution achieved at low pH; Good symmetry	Better separation, poor peak symmetry with large peak widths
Ammonium Carbonate	Good separation at pH 3.00. No base line separation at 2.14 and 4.41. Good peak shapes.	Improved resolution at pH 2.14 and 4.41. Best separation at pH 3.00.
Calcium Nitrate	No baseline separation at 2.14, 3.00 3.7 or 4.41. A change in selectivity is seen. Additional unknown peak is observed.	No baseline separation. Addition of an organic modifier gave good resolution at pH 3.00. Additional peak not observed.
Chromium Chloride	Separation is seen at 2.14 to 3.7. No baseline resolution at 4.41. Poor peak symmetry.	Resolution has improved and peak shapes improved.

Capillary Type	Results and Observations	
	Etched	Modified
Sodium Nitrate	Good resolution. Better peak symmetry at 3.7. Unknown peaks are observed.	Peak shapes have improved. Good resolution. Additional peaks are observed.
Modified with $CuCl_2$ not treated with 0.1M NaOH	Good and baseline separation is achieved at low pH values: 2.14, 3.00, 3.7 and 4.41. Good peak shapes.	
No Salts	Best separation with good peak shapes are observed at 3.00, 3.7 and 4.41. No baseline resolution is seen at 2.14 and 4.41. A little fronting is seen at pH 2.14	

Table 5: Results and Observations of Separation of Heterocyclic Aromatic Amines.

Capillary Type	Results and Observations	
Bare capillary	Resolution is observed at 2.14. Above pH 3.00 poor resolution is seen. At pH 4.41 the order of migration has changed.	
	Etched	Etched and C18 Modified
Cupric Chloride	Separation is seen at 2.14 and 3.00. No baseline separation for the first two peaks. Resolution lost at pH 3.7	Peaks unresolved. Five solutes co-migrated with no baseline separation

4. CONCLUSIONS

Chemical modifications of capillaries were carried out by the sequence of etching, silanization, and hydrosilation. The etching agent was saturated with an inorganic salt and the capillary was etched before modification with a C-18 group. Five inorganic salts that are soluble in methanol were used: cupric chloride; chromium chloride; calcium nitrate; sodium nitrate and ammonium carbonate. Hence five sets of etched only, etched and modified capillaries with each salt were prepared. The capillary inner surface dissolves in the etching agent and then precipitates back. This process results in an increase in the internal surface area of the capillaries up to 1000-fold. Along with this the ions of the salt present in the etching agent get incorporated into the surface of the capillary. Then a bulky octadecyl group is bonded via the formation of a hydride. The modification of the capillary with the bulky hydrophobic octadecyl group and the presence of inorganic salt led to selective interaction between the stationary phase and the solutes resulting in separations in some cases. The EOF is not negative in the presence of these inorganic salts as usually is seen in the etched chemically modified capillary without salts. In addition to this, differences in the migration times between the capillaries with different inorganic salts were observed. A change in the selectivity and the peak shape is also detected in some instances.

Though the process of converting Si-OH groups to Si-H moieties and later bonding an organic C18 group was found effective in preventing the

adsorption of basic compounds, the existence of the salts made some differences in the overall separation. The use of an organic modifier in the buffer did make a difference in the interaction of the sample with the surface of the capillary. The sample compounds might have also added to the differences in the observed behavior. In other words the mass of the peptides being similar, the pKa values of the peptides may be the crucial factor for the differences in the selectivity and migration times.

Heterocyclic amines were found to be successfully separated on bare and etched capillaries though absolute resolution was not achieved for MeIQ and IQ. The separation was not successful using the capillary etched with $CuCl_2$ and chemically modified, and the peak shapes are noticeably poor.

5. FUTURE STUDIES

Spectroscopic studies like X – ray photoelectron spectroscopy (XPS) are needed for further understanding of the fate of the salts during the course of etching. Employment of DRIFT spectroscopy for further characterization of the surface of the capillary would be helpful for understanding the extent of bonding that occurred in the presence of salts. Surface-Enhanced Raman Spectroscopy (SERS) may be helpful to understand whether the adsorption of peptides to the ions of surface matrix is taking place.

Elucidation of the chemical features of the etched surface and of the surfaces obtained after each silanization / hydrosilation reaction might be beneficial. Such characterization data might provide an explanation for the range of separation patterns obtained among the various etched and chemically modified capillaries. Further electrochromatographic studies have to be done on the capillaries. Such studies would involve the separation of biomolecules like proteins, peptides or inorganic compounds for a better understanding of the interactions of the solute with the stationary phase or the surface. This is needed to confirm that the results that were attained are purely due to the change in the surface matrix.

Mass spectroscopic studies are required to understand the extra unknown peak(s) that were observed in a few instances. These studies could be helpful to understand whether the unknown peaks are due to solutes interacting with the surface matrix or may be due to some impurities. There

are some drastic changes in the EOF of the etched and etched chemically modified capillaries with salts. Studies that give some information regarding the ionization, or the chemical nature of the inorganic salts in the presence of organic buffers might be helpful to understand the changes in the EOF.

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